

Reliable, Responsive Pacemaking and Pattern Generation With Minimal Cell Numbers: the Crustacean Cardiac Ganglion

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Abstract. Investigations of the electrophysiology of crustacean cardiac ganglia over the last half-century are reviewed for their contributions to elucidating the cellular mechanisms and interactions by which a small (as few as nine cells) neuronal network accomplishes extremely reliable, rhythmical, patterned activation of muscular activity—in this case, beating of the neurogenic heart. This ganglion is thus a model for pacemaking and central pattern generation. Favorable anatomy has permitted voltage- and space-clamp analyses of voltage-dependent ionic currents that endow each neuron with the intrinsic ability to respond with rhythmical, patterned impulse activity to nonpatterned stimulation. The crustacean soma and initial axon segment do not support impulse generation but integrate input from stretch-sensitive dendrites and electrotonic and chemically mediated synapses on axonal processes in neuropils. The soma and initial axon produce a depolarization-activated, calcium-mediated, sustained potential, the “driver potential,” so-called because it drives a train of impulses at the “trigger zone” of the axon. Extreme reliability results from redundancy and the electrotonic coupling and synaptic interaction among all the neurons. Complex modulation by central nervous system inputs and by neurohormones to adjust heart pumping to physiological demands has long been demonstrated, but much remains to be learned about the cellular and molecular mechanisms of action. The continuing relevance of the crustacean cardiac ganglion as a relatively simple model for pacemaking and central pattern generation is confirmed by the rapidly widening documen-

tation of intrinsic potentials such as plateau potentials in neurons of all major animal groups. The suite of ionic currents (a slowly inactivating calcium current and various potassium currents, with variations) observed for the crustacean cardiac ganglion have been implicated in or proven to underlie a majority of the intrinsic potentials of neurons involved in pattern generation.

A Physiologically Responsive, Reliable Heart Pacemaker and Muscle Activator Built From Nine Neurons

The crustacean cardiac ganglion (CG) is composed of from 6 to 16 neurons, 9 in most decapods, that autonomously provide rhythmically recurring barrages of action potentials to activate the heart muscle. In Malacostraca, the heart is neurogenic and in adults dependent for its beating on the impulses from the ganglion. The CG, consisting of the neurons and their processes, wrapped in glial and connective tissue, forms an elongated, discrete branching trunk in or on the heart. It can be dissected from the heart and will continue to show spontaneous, rhythmical bursting. As an accessible and robust *in vitro* preparation, the CG joins a list of crustacean preparations that have provided insights into fundamental neurophysiological mechanisms, in this case the mechanisms by which small neuronal networks can generate rhythmical, patterned output (reviews: Wiens, 1982; Marder and Calabrese, 1996). Possibly the most important contribution was the demonstration that individual neurons are endowed with an intrinsic burst-organizing mechanism that results in a patterned output to any appropriate excitatory drive, and the detailed analysis of the ionic mechanisms involved. Further, the CG demonstrated that interconnections among a small number of neurons with

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Abbreviations: CG, cardiac ganglion; DP, driver potential; PO, pericardial organ.

such a capability can ensure coordinated, patterned, rhythmic, highly fault-tolerant output from the ensemble. Patterned or bursting impulses are, of course, the essential effective activator of responses of other neurons or muscles or secretory cells. The recognition of patterning mechanisms intrinsic to individual neurons has simplified the analysis of neuronal network pattern generation, freeing it from seeking reliance on properties emergent from a network.

This review seeks to provide a reader with an overview of the studies on the CG in the context of crustacean heart function, with emphasis on the electrophysiological studies of the isolated CG. The ionic mechanisms observed have proven broadly applicable to explaining intrinsic burst generation or pattern generation by neurons in both invertebrate and vertebrate nervous systems (see further below). As early investigators noted (Welsh and Maynard, 1951), the CG has the essential properties of a brain: apparently autonomous spontaneity and the ability to sense relevant environmental changes (degree of heart filling, hormones) and integrate them with intrinsic pattern-generating properties to provide an appropriately adjusted motor output.

The unique suitability of the CG for analyzing cellular mechanisms for pattern generation and rhythmicity derives from particularities of crustacean neuronal functional anatomy, specifically the segregation of the major impulse-generating mechanism (voltage-dependent Na^+ conductance) to axons so that the soma and initial axon segment are not actively invaded by impulses ("spikes"). In the more elongate CG of lobsters, the well-separated distribution of the neuronal somata makes possible the physical separation of the impulse-generating axon or axons from the more subtle electrical responses of the non-impulse-generating soma, initial axon segments, and associated collaterals. One of these is a graded, regenerative, Ca^{2+} -mediated response to depolarization, lasting 200 ms or longer, which provides drive for generation of the burst of impulses by the axon and hence is referred to as a driver potential (DP). As reviewed below, the characteristics of DPs, which, in isolated CG, are initiated in response to spontaneously occurring pacemaker depolarization or to synaptic excitation, provide a basis for interpreting many of the responses of intact hearts as well as the isolated CG to both physiological and experimentally imposed modulatory influences (see also Cooke, 1988, 2002).

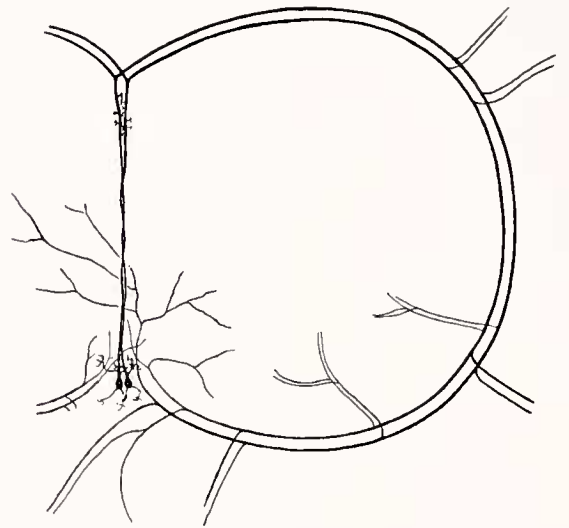
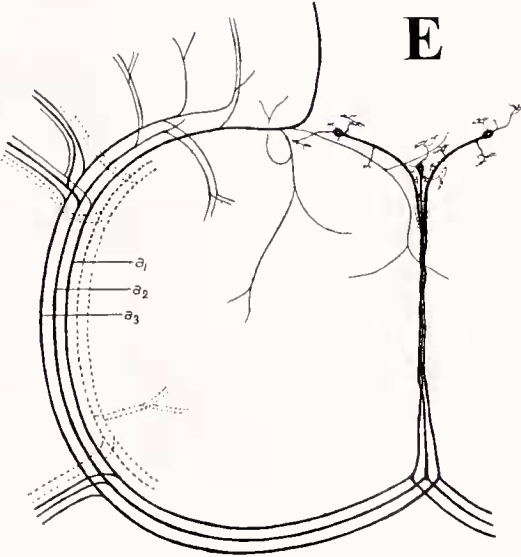
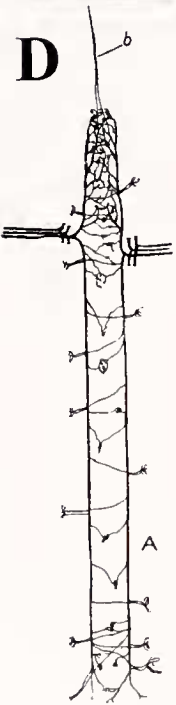
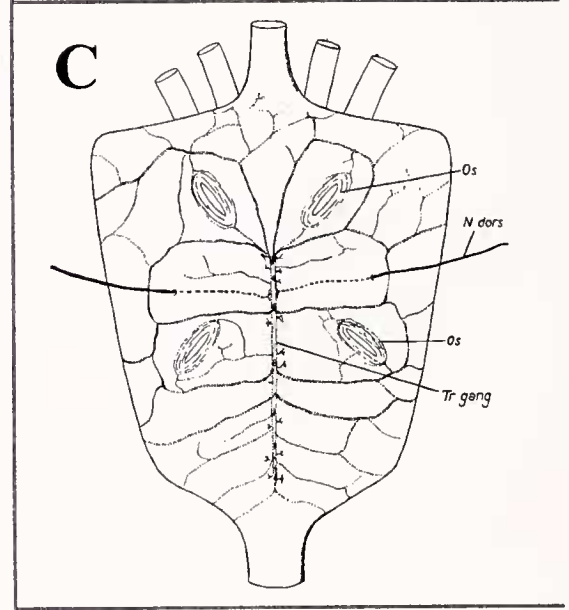
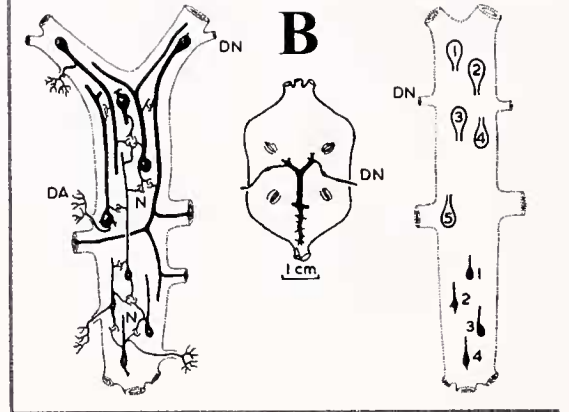
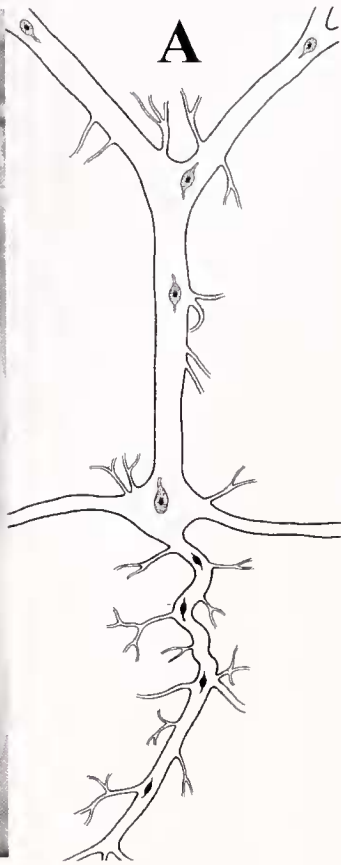
Anatomy

Morphological observations

Alexandrowicz (1932) provided an anatomical description of the innervation of decapod hearts that was based on vital methylene blue staining; this description has provided a structural underpinning for many subsequent observations of function (Fig. 1). Similar studies of the heart of stomatopods (1934) and of an isopod (1952) followed (see also

Suzuki, 1934). Alexandrowicz described three neuronal elements innervating decapod hearts: the intrinsic neurons (cardiac ganglion); the extrinsic fibers—one inhibitory and two acceleratory axons—arriving *via* three segmental nerves on each side from thoracic ganglia (combined into the dorsal nerves in decapods) to reach the heart and CG; and nerves innervating the suspensory ligaments, ostia, and arterial valves (Fig. 1B, C, D). He called attention to the neurogenic nature of the crustacean hearts, reporting the immediate cessation of contractions after nerves between the ganglion and muscle were cut. He also observed that the most anterior extrinsic nerve pair in stomatopods was inhibitory and the sequentially more posterior two pairs were excitatory, and correctly surmised that these have similar roles in decapods (Wiersma and Novitski, 1942, crayfish; Smith, 1947, crabs; Maynard, 1953, lobsters). Alexandrowicz provided important additional observations relevant to regulation of crustacean hearts: the anatomical description of the pericardial organs (POs) of crabs and their macruran homolog, the ligamental plexuses. These are neurohemal structures that release neurohormones having cardioregulator and other functions into hemolymph returning to the heart (Alexandrowicz, 1953; Alexandrowicz and Carlisle, 1953).

Comparison of the circulatory systems across the orders of Crustacea supports a consensus that evolution from a pulsatile dorsal vessel toward a compact heart accompanies more active lifestyles to provide for more efficient circulation (Wilkins, 1999; for a comprehensive review, see Maynard, 1960). In the primitive branchiopods (*e.g.*, *Daphnia* sp., *Triops longicaudatus*), no neurons have been found in or on the heart, and the heartbeat is thus myogenic (Yamagishi *et al.*, 1997). In the CG of the stomatopod *Squilla* (Alexandrowicz, 1934), 14 or 15 neurons are distributed along a ganglionic trunk that lies on the external dorsal surface of the heart and can be as long as 8 cm. In decapods, the CG lies on the inner dorsal wall of the heart (Fig. 1A, C). Although crayfish have 16 intrinsic neurons, in most decapods examined the number has been reduced to 9. There is a clear distinction in size and function. The most posterior four neurons are smaller and, having axonal terminations within neuropil in the ganglionic trunk, are therefore interneurons. The more anterior five neurons are larger and, because they provide axons that leave the ganglion to innervate heart muscle fibers, are motoneurons (Fig. 1B, E). Each of the neurons has dendritic or collateral processes extending out of the ganglionic trunk to ramify onto nearby muscle fibers that are responsive to stretch. In lobsters the somata are widely spaced along a linear (*e.g.*, *Panulirus*, Fig. 1C) or Y-shaped (*e.g.*, *Homarus*, Fig. 1A) ganglionic trunk spanning nearly a centimeter in a 0.5-kg animal; in crabs of edible size the neurons are usually compacted into anterior and posterior clusters separated by several millimeters of ganglionic trunk (Figs. 1E, 8D).



Electrophysiological anatomy

One of the first conclusions to come from electrophysiological recording from lobster CG (Welsh and Maynard, 1951; Maynard, 1955; see also Matsui, 1955) was that not only the rhythmicity but also the pattern of impulses within the bursts of activity is extraordinarily stable (Figs. 2C, E; 6A, B; 9E, F). In *Homarus americanus*, for example, output of isolated CG consists of a 200- to 300-ms burst of tightly grouped efferent impulses that recur spontaneously at rates similar to observed heartbeat rates (50–60/min for lobsters). Further confirmation of the consistency of burst patterning came with analysis of the patterning by Hartline (1967), who used an array of five or more pairs of extracellular electrodes placed along the trunk and major nerves of the *Homarus* ganglion to identify each impulse with its axon by mapping the site of impulse initiation and its conduction route (Fig. 2A, B, D). It was clear that a particular one of the posterior cells (usually 8 or 7, numbering cells from anterior to posterior) consistently fired first in a burst, and that the firing of large cells commenced with the arrival of the first small-cell impulse, propagated along its anterior-traveling axon, at neuropil in which synapses on large-cell collateral processes occur. Thus the most posterior large cell (5) fired first, followed successively by more-anterior cells. The sites of impulse initiation observed in such studies confirmed conclusions reached from intracellular recording from the large cells (early work reviewed by Hagiwara, 1961) that impulses do not invade the somata, but rather are initiated at a site that can be more than a millimeter distant along the axon. Cells having more than one axon—for example, Cell 3 situated at the junction of the Y in *Homarus*—initiate impulses in each axon independently (Fig. 2D). As mentioned above, within each burst, the patterning of impulses of each axon remains highly constant, each unit showing repetitive firing (Fig. 2C). In *Homarus*, the first 3 to 4 impulses of large-cell axons occur

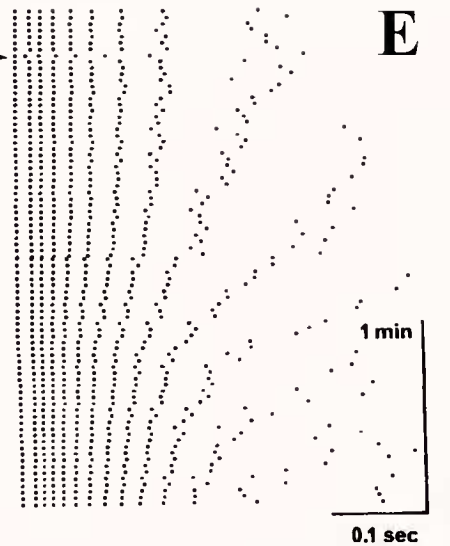
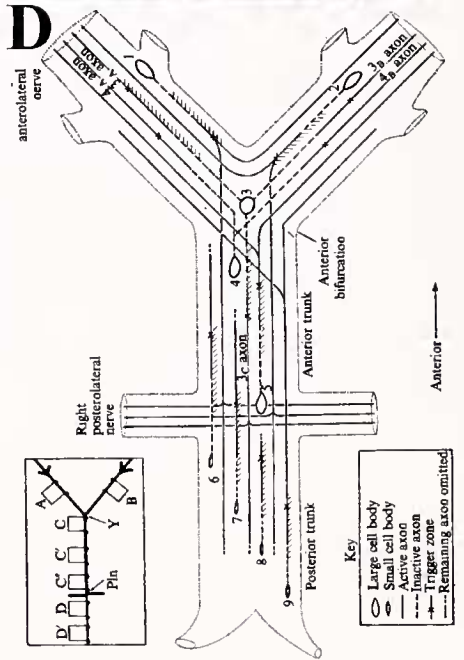
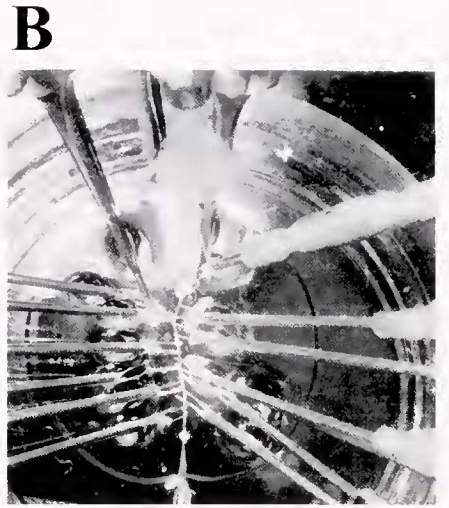
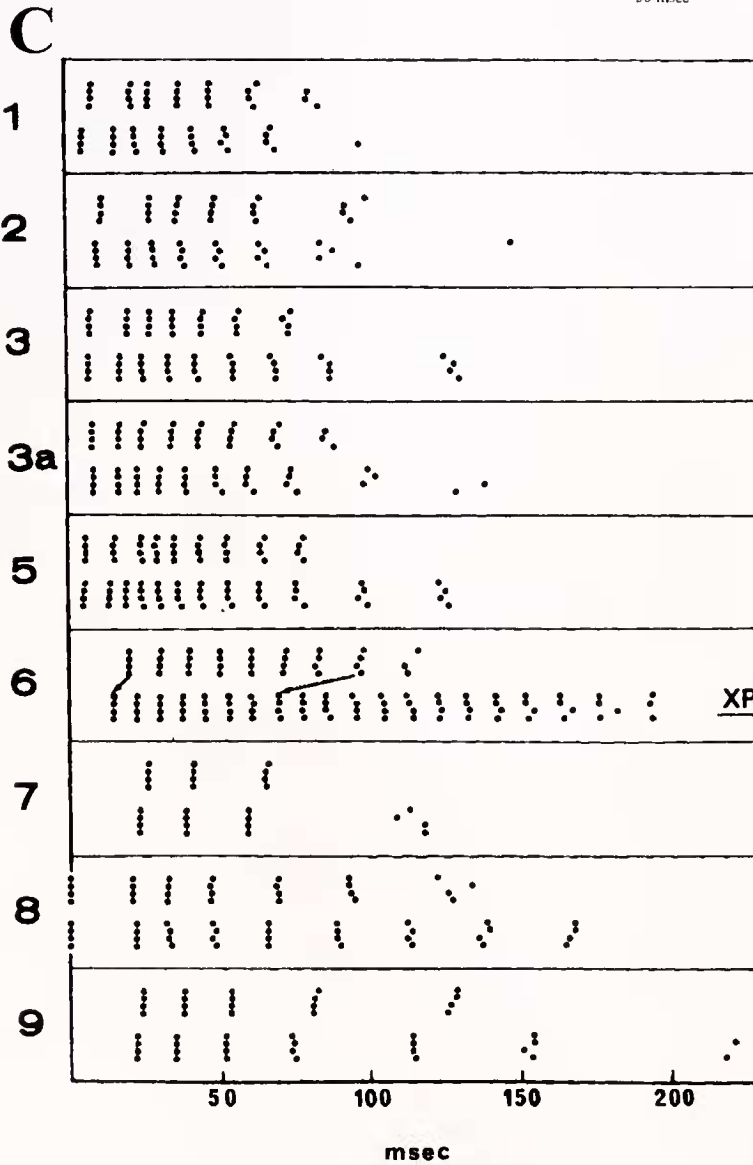
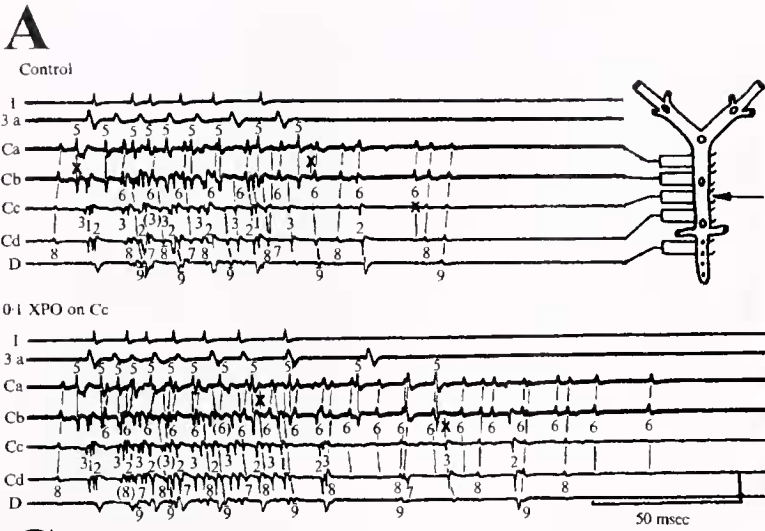
at high frequency (90–120/s) and then continue for 3 or more at a slower rate (10–20/s, Hartline, 1967); small-cell axons fire as many as 15 impulses starting at rates of ~80/s and declining during the burst toward 20–30/s. A similar analysis showing minor differences in detail from *Homarus* is available for *Panulirus interruptus* (Friesen, 1975a, b).

Studies of functional anatomy in crab CG (Tazaki and Cooke, 1979a, 1983a, in *Portunus sanguinolentus*; Fort and Miller, 2001, in *Callinectes sapidus*) show an important variation on this organization, namely that large-cell axons, rather than each firing in a consistent individual pattern within each burst, show synchronization of their impulse firing (Fig. 6A). Synchronization also occurs among the rostral neurons of the *Squilla* ganglion (Watanabe and Takeda, 1963).

Axons of the posterior four small cells in the lobster and crab CG remain within the ganglionic trunk and provide excitatory, chemically mediated synaptic input to the large cells. This input initiates burst activity of the large cells, and thus the small cells are considered pacemaker interneurons or premotor neurons. The large-cell axons, while providing synaptic input to each other and perhaps also to the small cells, produce the bursts of motor impulses responsible for heart muscle contraction. Synchronization of ganglionic activity is not only mediated by synaptic drive, but the general excitability of the network is shared among all of the neurons by means of electrotonic coupling capable of passing slow changes in potential. Synaptic and electrotonic interactions are discussed below.

Two further properties of the individual CG neurons are also critical: each has stretch-sensitive dendrites ramifying into heart muscle that account for the ability of intact hearts to adjust heart rate and strength of beating to the degree of filling of the heart; and each has the intrinsic ability to produce a patterned burst of impulses in response to a simple stimulus.

Figure 1. Anatomy of the crustacean cardiac ganglion. Anterior up. (A) Fresh dissection of a *Homarus americanus* cardiac ganglion exposed on the inner dorsal surface of the heart (length of field ~1.3 cm); diagram shows the approximate position of the nine intrinsic neurons (for nomenclature see Fig. 2D). Extrinsic regulator nerves (dorsal nerves) enter laterally just anterior to the most anterior neurons. (Cooke, 1962, fig. 1-1.) (B) Cardiac ganglion (diagrammatic). Left, *Homarus*: relations of the cell bodies, neuropils (N), and dendritic arborizations (DA). Full course of axons is not shown. DN, dorsal nerve. Right, *Panulirus*: positions of cell bodies and direction of major axons. Inset, *Homarus* ganglion trunk *in situ* in heart. Length:width ratio much reduced in all diagrams. (Maynard, 1955, fig. 1.) (C) Alexandrowicz' semi-diagrammatic representation of the nervous system in the dorsal wall of the heart of *Palinurus vulgaris*. *Tr gang*, ganglionic trunk, with its nerve-cells: *N dors*, dorsal nerve piercing the heart-wall; *Os*, ostium. Alexandrowicz' drawings compiled repetitive observations of fresh dissections during methylene blue vital staining. (Alexandrowicz, 1932, Text-fig. 1.) (D) Diagram showing the course of the fibers of System I [inhibitors] of the dorsal nerves in *Palinurus*. Only one fiber on each side is represented. *b*, fibers of unknown destination arising from fibers of System I. (Alexandrowicz, 1932, Text-fig. 19A.) See also Fig. 7F. (E) Diagram illustrating the course of the axons of three anterior cells (left half) and two posterior cells (right half) in *Cancer pagurus*. In dotted line are drawn parts of the axons of posterior cells. (Modified from Alexandrowicz, 1932, Text-figs. 8, 9.) (Figures in C, D, and E from Alexandrowicz, 1932, are by permission of Company of Biologists, Ltd.)



An Intrinsic Burst-Forming Mechanism: the Driver Potential

Possibly first discussed as an *intrinsic* potential by Watanabe (1958) in lobster CG, and further described in studies of the *Squilla* (stomatopod) CG (Watanabe *et al.*, 1967a, b), driver potentials are relatively slow, sustained, regenerative depolarizations that may arise from a gradual pacemaker potential or be evoked by a depolarization, such as an excitatory synaptic potential (or an applied depolarizing stimulus) (Fig. 3). They provide the depolarizing drive for initiating repetitive impulses at an axonal "trigger zone." Because DPs arise in the soma and proximal axon or axons—regions lacking impulse-generating conductances and, particularly in lobster CG, physically well separated from spiking axon—they can be studied in relative isolation. Their properties account for much of the collective behavior of the network, including rhythmicity, reciprocity between burst rate and duration, and phase resetting in response to imposed extra stimuli.

Driver potentials arise in the non-spiking soma and proximal axon

Direct evidence for the localization of DPs is provided by simultaneous intracellular recording from a neuronal soma and its axon at a distance of several millimeters (Fig. 3A): the soma recording shows a sustained, slow depolarization (*i.e.*, the DP) with attenuated sharp deflections that are synchronous with the overshooting impulses arising from a

flat baseline recorded from the axon (Watanabe *et al.*, 1967b, *Squilla oratorio*; Tazaki, 1970, *Eriocheir japonicus*; Tazaki, 1973, *Panulirus japonicus*; Tazaki and Cooke, 1983a, *Portunus sanguinolentus*). The localization of DPs to the soma and non-impulse-supporting initial axon was also shown in *Homarus* by intracellular recording during or after ligaturing at distances between 200 μm and more than a millimeter from the soma (Tazaki and Cooke, 1983b) (Fig. 3B). For more distant ligatures, electrotonically decremented impulses, as recorded from the soma, were superimposed on the DP, indicating that the ligatured segment included an axonal trigger zone. It is worth noting that more complex deflections, suggestive of synaptic potentials, were sometimes present in recordings from ganglion segments that include a single soma. This suggests the possibility that the processes of other neurons present in such a segment can contribute synaptic input. Any rapid deflections disappeared with only minimal change in the form of the underlying DP when tetrodotoxin (TTX) was added to the perfusing saline (Tazaki, 1971a). Treatment of ganglia with TTX made it possible to observe DPs in the absence of any impulse-mediated activity by simultaneous intracellular recording in up to three large cells (Tazaki and Cooke, 1979b, in *Portunus*) (Fig. 3D, two cells). Stimulation with a depolarizing current pulse in any one cell simultaneously initiated DPs in all; the amplitudes and form differed slightly, but remained characteristic in each cell, hence indicating that the DP represented an active response of each neuron, but one that was brought to threshold by the spread of the depolarizing

Figure 2. Electrophysiological anatomy: responses to localized neurohormone application. (A) The site of origin of impulses, their route of propagation and recurrence are analyzed by simultaneous recordings from seven pairs of electrodes (selected from 11, see B) placed on an isolated *Homarus* cardiac ganglion. Numbers identify impulses of corresponding cell axons ($3 = 3c$) as indicated in D. X indicates site of impulse initiation; bar over cell number indicates distally initiated impulse; brackets indicate uncertainty in identification or timing. The lower records show the response to application at the position indicated by the arrow of a droplet ($\sim 10 \mu\text{l}$) under the mineral oil of pericardial organ extract (XPO, POs from two *Cancer borealis*, 0.5 mg dry weight in 1 ml, heated to 100°C). (B) Photograph of a *Homarus* ganglion with electrodes positioned for extracellular recording as in A. The ganglion is lifted out of saline into mineral oil for recording. (C) "Dot pattern" analysis of the records. For each of eight bursts (four control; four experimental), including the two from A, firing times for each unit are indicated as a horizontal line of dots. Beneath the first burst pattern for a unit, the succeeding three burst patterns for that unit are positioned relative to the pacemaker impulse in Cell 8. Note the typical consistency in firing pattern of a unit within the four bursts. The arrow indicates the change of firing position of the last stable Cell 6 impulse during the response. The effect of the XPO application has been to increase the frequency and number of impulses of Cell 6 selectively and to contract its firing pattern. (D) Diagram of locations, axon courses, and locations of anatomical regions for the nine intrinsic neurons. Active axon (refer to key) is axon carrying a regenerative impulse. The shaded portion between soma and trigger zone represents a region of pharmacological sensitivity. Note that Cells 3 and 4 have more than one independent axon. Width of ganglion exaggerated relative to length (total length about 1 cm in 0.5-kg animals). Inset: Designations given to locations along the ganglion used in identifying electrode and drug placements. (E) Effect on impulse firing pattern of application of XPO to the proximal axon of Cell 5. Axon 5 impulses brighten the oscilloscope beam, which is triggered by the first impulse of each burst and swept horizontally while also being continuously slowly displaced downward. Application was made after the seventh burst (arrow and electrical artifact). The average firing frequency of Cell 5 was increased 38%, but coordinated by addition of impulse firing within bursts without a change of burst rate (dot rows remain evenly spaced). (A, C, fig. 2; D, fig. 1; E, fig. 4 from Cooke and Hartline, 1975; by permission of Company of Biologists, Ltd. B, Cooke and Hartline, unpublished.)

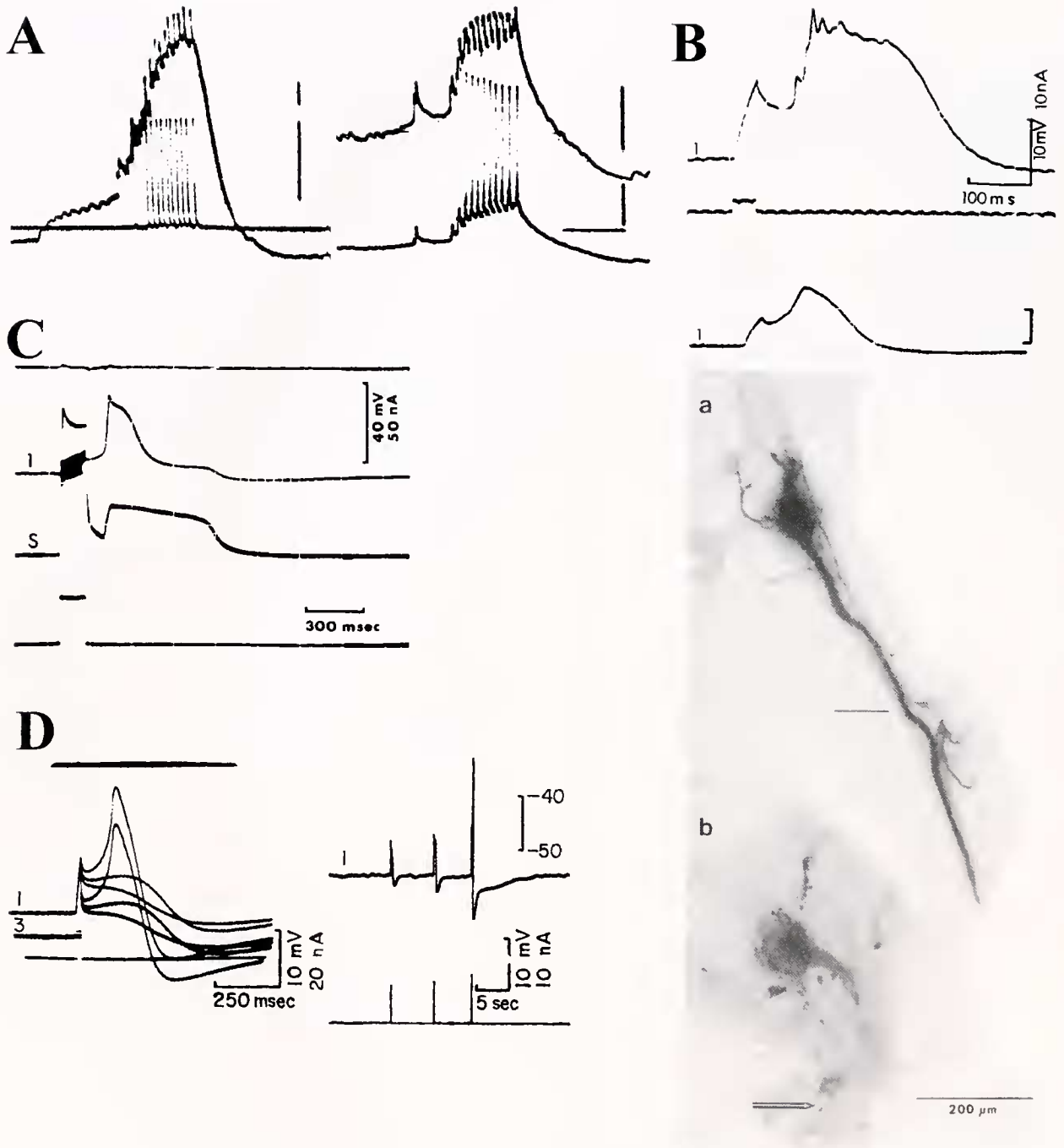


Figure 3. Localization of the driver potential to the soma and proximal axon. (A) Spontaneous bursts of spikes recorded simultaneously from two regions of a large neuron of the crab *Eriocheir japonicus*. Left frame: upper trace, axon; lower trace, soma; distance between them, 2.5 mm. Right frame: upper trace, soma; lower trace, axon; distance, 0.3 mm. Calibration: 10 mV, 100 ms. Note lack of underlying depolarization in distal axon, presence in axon near soma, attenuated impulses in soma. (Tazaki, 1970, fig. 1.) (B) Depolarization-evoked responses of neurons ligatured at different distances (*Homarus*). Upper frame: intracellular recording from Cell 1 ligatured 1.6 mm from the soma (a, below); response to a depolarizing current pulse passed through the recording electrode evokes a driver potential on which are superimposed more rapid, small deflections, interpreted as electronically decremented impulses of the axon and possible other elements present in the ganglion segment. Resting potential, -54 mV. Lower frame: response recorded from Cell 1 ligatured 200 μ m from the soma (b, below); a driver potential without superimposed deflections is observed. Calibration mark, 10 mV, 10 nA. Resting potential, -50 mV. Photomicrographs (reversed image) of the neurons after Lucifer yellow

stimulus *via* pervasive electrotonic coupling among all neurons of the ganglion.

The form of their driver potential shapes the pattern of impulses of each neuron

Large cells. The DPs as recorded intracellularly from large cells of crabs or lobsters show little difference in form whether examined in TTX or after isolation of a soma by ligaturing. If the neuron is relatively undamaged (as evidenced by high input resistance and a resting potential ~ -50 mV), depolarization to a threshold (~ -45 mV) initiates a regenerative response requiring 10 or more ms to reach a maximum ~ 20 mV depolarized from resting potential (Fig. 3D). The depolarization shows a rounded peak with a gradually declining shoulder, followed by a more rapid repolarization that gives way to hyperpolarizing afterpotentials. These have a relatively rapidly decaying phase lasting up to 1 s, followed by a slowly decaying phase lasting tens of seconds. Although they are regenerative, DPs are not all-or-none responses; rather their size is related to the amplitude and rate of rise of the depolarizing stimulus. More importantly, threshold and amplitude are related to the rate of repetition: given a constant stimulus, threshold becomes lower and amplitude and duration become larger with increasing time since the previous response. A maximal response requires a pause of more than 10 s. It will be obvious that at typical heart rates of 50–60/min, DPs are not at maximal amplitude but are evoked in a range over which changes in heart rate will result in decreased or increased amplitudes (Fig. 5A). Possible cellular mechanisms governing these relations are discussed below.

Small cells. An intracellular recording from a small cell, simultaneously with a large cell, in a *Portunus* CG treated with TTX (Tazaki and Cooke, 1983c) reveals clear differences in the form of DPs of large and small cells (Fig. 3C). The DP in the small cell is lower in amplitude but has a long (~ 400 ms), sustained, slowly declining plateau. The initiation of the small-cell DP by depolarizing current also causes

a DP in the anterior large cell; this has the larger but less sustained form described above. The electrotonically spread continuing plateau of the small-cell DP is apparent in the large-cell recording. As mentioned previously, the bursts of the small cells are longer and show a well-sustained frequency of firing. Thus, the differing form of the DPs of small and large cells accounts for the differing pattern of impulses produced during bursts by the axons of small and large cells (Fig. 6A).

The ability of slow potentials such as DPs to generate trains of impulses from the axonal trigger zone implies that the ionic mechanisms involved in impulse initiation are not subject to rapid inactivation by depolarization. Intracellular recordings from axons, as mentioned above, show typical overshooting, all-or-none impulses with a rapid rise and fall. When intracellular penetrations were attempted, damage to the target neuron was often signaled by appearance in an extracellular recording of repetitive firing not organized into the coordinated bursting of the remaining cells of the ganglion, confirming the ability of axons to respond to sustained depolarization with minimal adaptation.

Voltage-Clamp Analyses of Ionic Currents Giving Rise to Driver Potentials

Characterization of four kinds of current in ligatured somata

The characteristics and ionic conductances responsible for DPs have been examined in most detail in the *Homarus* CG (Tazaki and Cooke, 1983c, 1986, 1990), in which the separation of the neurons permitted the isolation of ganglion segments with a single large-cell soma by ligaturing the ganglionic trunk. Studies with two-electrode voltage clamping show that DP characteristics involve the interplay between an inward Ca^{2+} -mediated current (I_{Ca}) and three outward K^{+} -mediated currents: a transient current (I_{A}), a slowly-inactivating K^{+} current (I_{K}), and a Ca-dependent K^{+} current (I_{KCa}) (Tazaki and Cooke, 1986, 1990). Space-

injection (glycerin-cleared whole mount photographed under dark-field UV illumination immediately after dye injection; scales, 200 μm). Ligature in a was just beyond stained end of axon; at arrow in b. Note fine processes and a major axon collateral proximal to the ligature in a. (Modified from Tazaki and Cooke, 1983b, Figs. 6, 7; by permission of Springer-Verlag CmbH & Co. KG.) (C) A driver potential response of a small cell in a *Portunus sanguinolentus* ganglion perfused with $3 \times 10^{-7} \text{M}$ TTX and 50 mM TEA. A depolarizing current pulse applied through the recording electrode in the small cell evokes a driver potential in both the small cell (S) and anterior Cell 1. The driver potential in cell 1 is followed by a low-amplitude potential representing the electrotonically conducted, longer-duration response of the small cell. Resting potentials, -48 mV. (Tazaki and Cooke, 1983c, fig. 8.) (D) Graded driver-potential responses. At the left, three superimposed sweeps show responses recorded intracellularly from Cells 1 and 3 (and absence of responses in extracellular electrode, top trace, $3 \times 10^{-7} \text{M}$ TTX in perfusate), to different intensities of 20-ms depolarizing current pulses applied *via* a bridge in Cell 3. Lowest trace is a current monitor. At the right, Cell 1 responses to the same current pulses recorded simultaneously on the penwriter, current monitor below. Responses are graded with current intensity below a threshold; above the threshold, responses are constant (not shown). Slow afterpotentials are observed only following above-threshold responses. Resting potentials, -55 mV. (Tazaki and Cooke, 1979b, fig. 3.)

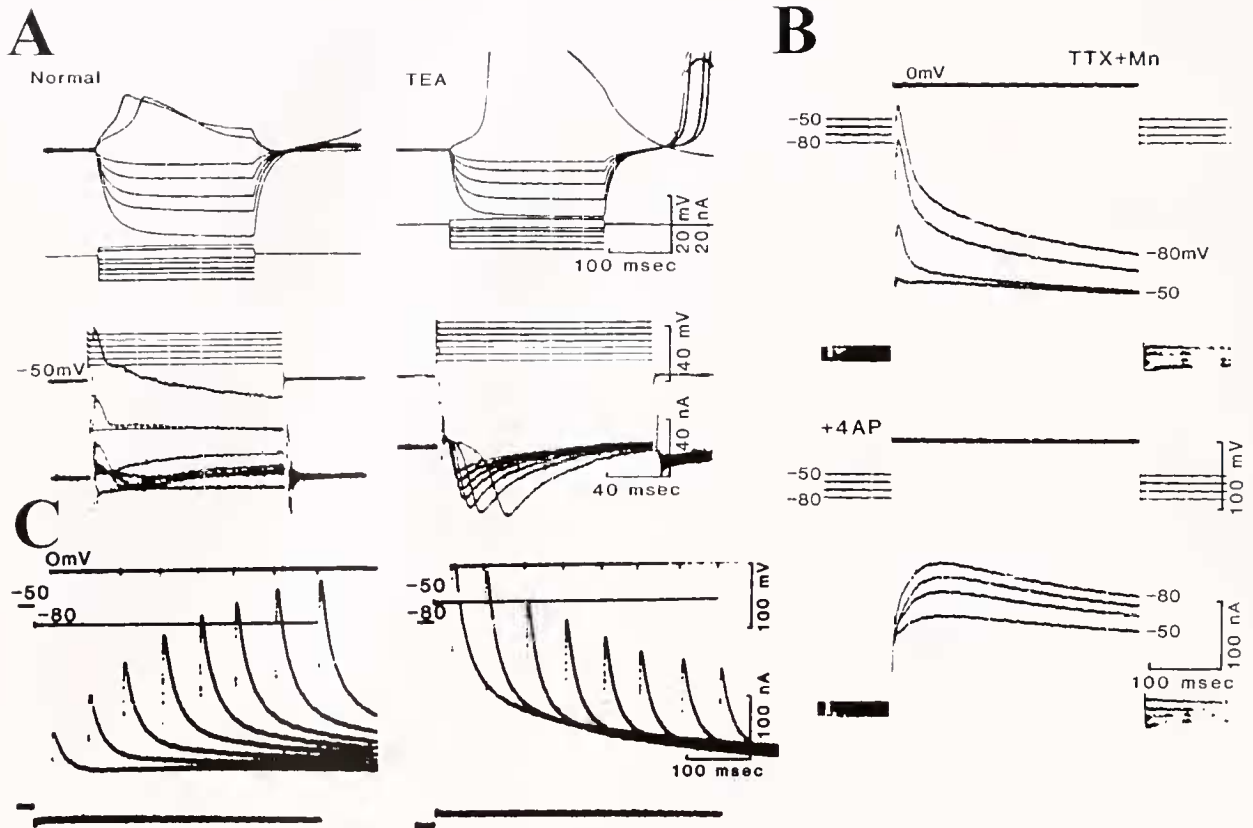


Figure 4. Voltage clamp analyses of ligatured *Homarus* cardiac ganglion large cells. (A) Responses of Cell 1 ligatured 200 μm from the soma and penetrated with separate recording and current-passing electrodes (upper traces, voltage; lower traces, current). Upper frames: responses to current clamps. Note the slowly increasing response to hyperpolarizing current. Depolarizing current produces driver potentials; in TEA (50 mM) their peak reaches 0 mV (as recorded from response to a brief pulse, not shown), and their duration is increased. Driver potentials are generated on cessation of hyperpolarizing current. Lower frames: membrane current responses (superimposed oscilloscope sweeps) to voltage clamps from -50 mV to successively more depolarized commands (left, -40 to -15 mV; right, -10 mV). In normal saline, inward current is indicated by downward notches, but outward currents dominate. A hyperpolarizing command (to -85 mV) indicates extent of leakage current. In TEA, outward current is largely inhibited, revealing inward current having nearly maximal amplitude but long latency at the smallest command. Resting potential, -50 mV. (B) Transient outward current (I_A) is selectively inhibited by 4-aminopyridine (4AP), leaving slowly inactivating outward current (I_K). Frames showing superimposed membrane current responses to voltage clamps from different V_h (-50 to -80 mV) to 0 mV in TTX ($3 \times 10^{-7} M$) and Mn (4 mM) (above) or in TTX, Mn, and 4AP (4 mM) (below). Note the inhibition of the initial outward current peak in 4AP. Cell ligatured at 1.5 mm (resting potential, -45 mV). (C) Effect of conditioning clamps on I_A . In each frame, eight sweeps are superimposed; for each, the conditioning clamp remains on longer before evoking outward current by clamping to 0 mV. Left frame: effect of hyperpolarizing conditioning clamp from $V_h = -50$ to -80 mV. Rate and amount of I_A augmentation increase with increased hyperpolarization (not shown). Right frame: effect of subthreshold depolarizing conditioning clamp from $V_h = -80$ to -50 mV. The rate and magnitude of the decrease in I_A increase with increasing depolarization (not shown). Rate of onset and decay of individual responses is not affected by subthreshold potential changes. TTX ($3 \times 10^{-7} M$) present throughout; cell ligatured at 1 mm (resting potential, -45 mV). (A, fig. 10, Tazaki and Cooke, 1983c; B, fig. 8 (modified), C, fig. 9 (modified), Tazaki and Cooke, 1986.)

clamp could be ensured in the *Homarus* ganglion by ligaturing. Without detailing the evidence, a brief summary of the conclusions from voltage-clamp analyses follows (see also Figs. 4 and 5 and legends). The depolarizing current that generates DPs arises from voltage-gated increased conductance to Ca^{2+} and the resulting inward Ca^{2+} current (I_{Ca}) (Figs. 4A, 5B). The amplitude of the DP is determined

by the extent of Ca^{2+} channels available for activation. An examination of the effect of the holding potential (V_h) of the large-cell on the amplitude of I_{Ca} shows a maximum for a potential (-60 mV) close to the most hyperpolarized value observed during the afterpotential following a burst. Peak current is reduced to half or less at more hyperpolarized V_h . The amplitude of the DP is normally limited by the nearly

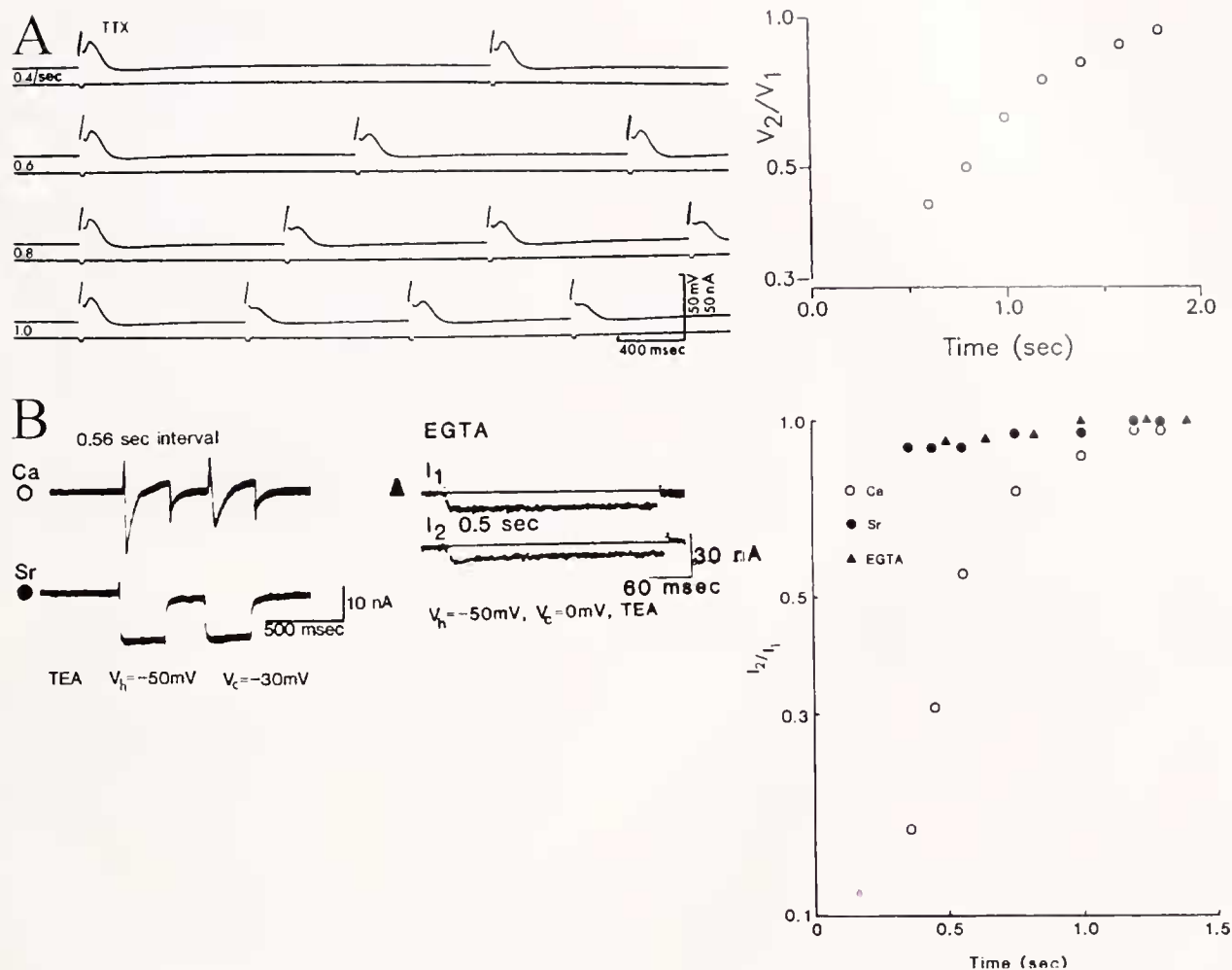


Figure 5. Characteristics of Ca^{2+} current influence driver potential form (*Homarus* large-cell somata, isolated by ligaturing). (A) Change of driver potential form with change of stimulus rate. Brief (25 ms) depolarizing current pulses (3 nA, depolarization downward, bottom trace of each pair) passed through an intracellular electrode elicit regenerative responses recorded with a second intracellular electrode in Cell 2 (resting potential, -55 mV). TTX ($3 \times 10^{-7} M$) is included in the superfused saline. Stimulus rate indicated for each pair of traces (moving photographic paper records from oscilloscope); first response follows an unstimulated interval of ≥ 30 s. Plot of the amplitude of the second response relative to the first (fully recovered) response vs. interval. At stimulus rates corresponding to rates of spontaneous bursting, driver potential responses are graded in amplitude. (B) Recovery of I_{Ca} from inactivation. Net inward current in response to an identical second clamp step given at varied intervals is compared with that to the first, given after ≥ 30 s rest. Left traces: examples of responses of the same isolated soma in standard and in Sr-substituted saline. Right traces: responses of another soma that had been injected with EGTA. Note that in contrast to the control records, the second responses in the absence of an increase in $[\text{Ca}^{2+}]_i$, are nearly equal to the first and show little decline of inward current during clamp steps. Semilogarithmic plot of normalized second response amplitude vs. interval for the somata providing the records shown above. Initial recovery in Ca saline occurs with $\tau \sim 640$ ms; complete recovery requires several seconds. (A, fig. 1, B, fig. 14, Tazaki and Cooke, 1990.)

simultaneous development, in response to depolarization, of the I_{K} conductance. If I_{K} is blocked with tetraethyl ammonium (TEA), I_{Ca} can produce overshooting potentials (Fig. 4A). Repolarization, and thus the duration of DPs, is the result of the interplay of I_{K} , inactivation of I_{Ca} primarily from intracellular accumulation of Ca^{2+} (Fig. 5B, D), and development of the Ca^{2+} -dependent I_{KCa} .

The voltage-clamping data are consistent with the major role of Ca^{2+} in mediating the DP depolarization (Figs. 4A, 5). However, a possible role of Na^{+} in contributing to inward current remains unclear, with conflicting observations of the effects of its reduction or removal from the saline (Tazaki and Cooke, 1979c; Berlind, 1985, 1993). The differences may be attributable to the manner of substitution

for Na^+ and to other differences in the experimental conditions.

The more rapidly decaying phase of the hyperpolarizing afterpotential following a CG driver potential (Fig. 3D) represents the deactivation of I_K with repolarization, while the slowly declining phase of the afterpotential probably reflects the sequestering or extrusion of Ca^{2+} to reduce I_{KCa} . A refractory period for initiation of DPs is imposed by a combination of the inactivation of I_{Ca} by intracellular accumulation of Ca^{2+} as well as the increased K^+ conductance from the Ca^{2+} -activation of I_{KCa} . When Na^+ -mediated impulses are present (in non-TTX-treated preparations), a hyperpolarizing current from activation of electrogenic ion transport also contributes to the afterhyperpolarization (Livengood and Kusano, 1972; Livengood, 1983). With time, the threshold for response to a depolarizing stimulus decreases (1) due to progressive reduction of the ability to activate transient K^+ conductance (I_A) with decline of the afterhyperpolarization or pacemaker depolarization (or both); and (2) with progressing Ca^{2+} extrusion or sequestration, reduction of the I_{KCa} conductance and of the Ca^{2+} -mediated inactivation of I_{Ca} due to previous activity (Fig. 5B).

Driver potentials and similar intrinsic patterning potentials have proven to be a more labile response than generation of action potentials. This is easily explained by the relatively smaller current densities involved and the closely balanced interplay of conductances that govern their form. In crustaceans, for example, I_{Ca} observed under voltage-clamping conditions can be inactivated by raised intracellular Ca^{2+} . Thus it is not surprising that crustacean DP generation fails if neural damage causes Ca^{2+} entry from the saline, decreases input resistance, depolarizes the cell membrane (thereby increasing I_K), and increases leakage conductance. That DPs represent a delicate balance in the interplay of inward and outward currents is confirmed by observing the spontaneous initiation of DPs in previously quiescent preparations and the augmentation of their amplitude and duration after addition of TEA to inhibit I_K (Tazaki and Cooke, 1979c; Berlind, 1993) (see below for further discussion of spontaneity).

Changes of DP amplitude and duration with repetition rate are determined by characteristics of I_{Ca}

Analysis of the pharmacologically isolated Ca^{2+} current in ligatured somata suggests that its characteristics dominate the determination of DP amplitude and duration during repetitive activation. Figure 5 combines observations (from Tazaki and Cooke, 1990) on DP amplitude vs. DP repetition rate (A), and rates of recovery of I_{Ca} for the second of two stimuli (B). These follow closely parallel time courses; the slightly faster recovery of I_{Ca} studied with a pair of pulses is explained by a cumulative inactivation of I_{Ca} that occurs

with repetitive activation. The role of intracellular Ca^{2+} accumulation as the main agent of inactivation is evidenced by the lack of inward current inactivation when Sr^{2+} substitutes for Ca^{2+} or the soma is injected with EGTA (Fig. 5B).

The accumulation of intracellular Ca^{2+} also activates I_{KCa} . This current is of small magnitude relative to I_{Ca} and I_K , and it influences primarily the rate of repolarization after a DP, or of a pacemaker depolarization during the equivalent of interburst intervals (Tazaki and Cooke, 1986).

Rhythmicity of Cardiac Ganglion Electrical Activity

The importance for survival of the animal of a reliable, rhythmic heartbeat cannot be exaggerated. The isolated cardiac ganglion, as the pacemaker and activator of heart contraction, has proven capable of sustaining robust, rhythmic motoneuron bursting when challenged with a variety of insults and perturbations.

Behavior of the cardiac ganglion as an oscillator

A number of studies demonstrate that small sustained currents passed into a large CG soma can alter the coordinated burst rate of the entire ganglion, while brief pulses of hyperpolarizing or depolarizing current can reset the bursting phase (Fig. 6A) (Watanabe and Bullock, 1960; Watanabe *et al.*, 1967b, in *Squilla*; Tazaki, 1972, in *Eriocheir*; Mayeri, 1973a, b, in *Homarus*; Matsui *et al.*, 1977, in *Panulirus*; Tazaki and Cooke, 1979a, and Benson, 1980, in *Portunus*). The coordinated response of the entire ganglion to such perturbation of a single neuron is ensured by the combination of electrotonic and probably reciprocal excitatory synaptic interactions among all the cells. The characteristics of I_{Ca} that determine the threshold and amplitude were discussed above and help to explain the reciprocity observed between burst rate (or interburst interval) and duration.

Autonomous rhythmicity: stretch responsiveness and pacemaker potentials

The basis of the spontaneity exhibited by isolated CG preparations is considered in this section. A question arises from the observation that an intact heart, if not still suspended by its elastic ligaments or stretched by internal perfusion, rapidly becomes quiescent. *In situ*, expansion and filling of the heart, mediated by the suspensory ligaments, probably aided under some conditions by contraction of alary muscles, stimulates heart contraction. The response to stretch or filling is undoubtedly mediated by the dendritic and collateral processes of the CG cells that ramify on heart muscle near the ganglion. Alexandrowicz (1932) pointed out that the terminations of these processes differed from the more peripheral neuromuscular junctions and noted their

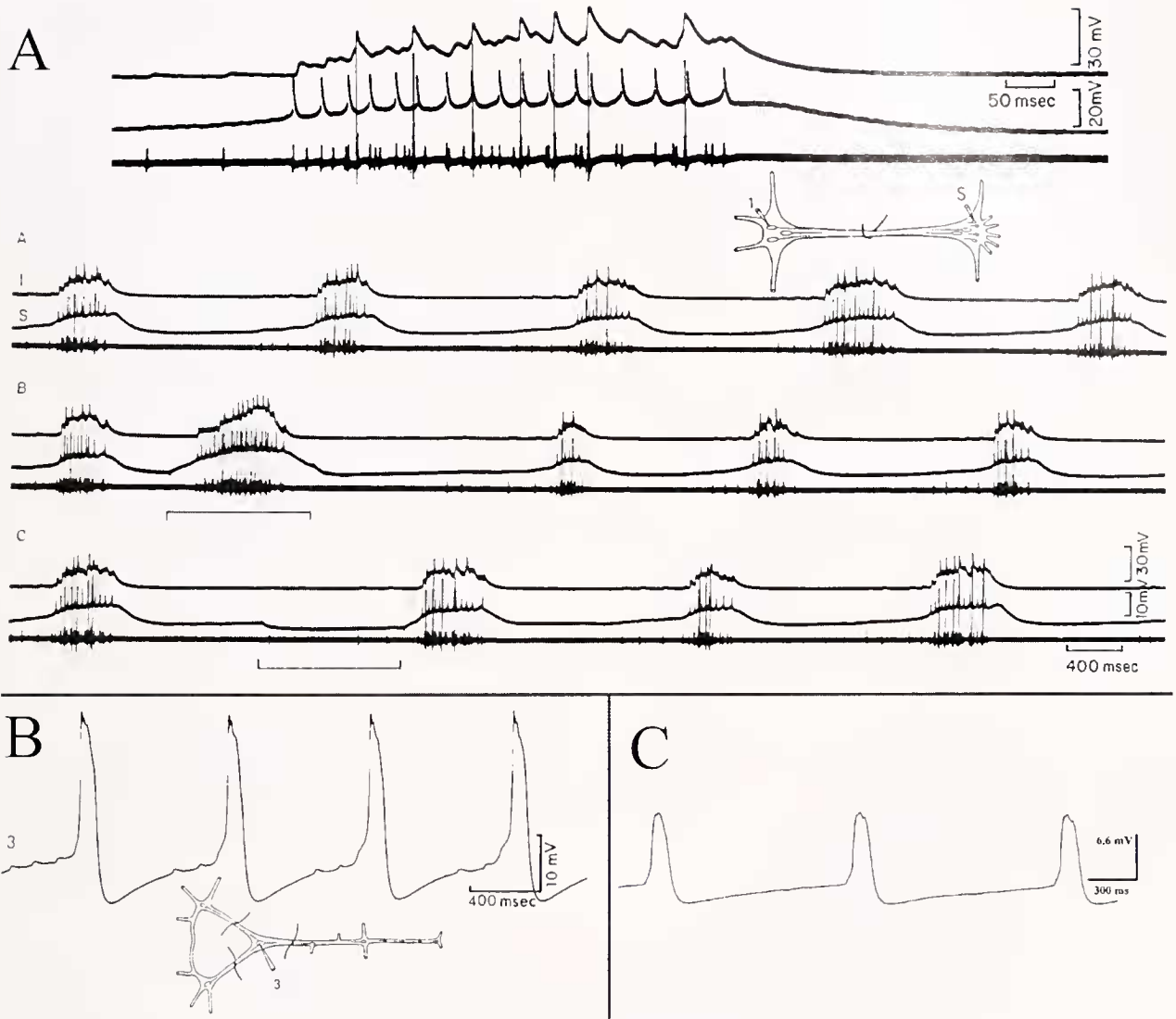


Figure 6. Spontaneous bursting characteristics. (A) Spontaneous bursting of the isolated *Portunus sanguinolentus* ganglion recorded intracellularly from a small cell (S), from an anterior large cell (1), and extracellularly (see inset). In the burst shown at expanded time-base (top), the correspondence between rapid depolarizations in the small cell, a small impulse in the extracellular record and, following a delay, a synaptic potential in the large cell may be observed. Very large spikes in the extracellular record (retouched) represent the synchronous firing of large cell axons and are seen as decremented impulses in the large soma. Records below show A, spontaneous bursting; B, the effect of passing depolarizing current (0.8 nA, upward bar) or C, hyperpolarizing current (0.8 nA via a bridge into the small cell). In B, note the premature initiation of a driver potential and burst in the large cell after extensive synaptic driving by the small cell, and the compensatory following delay and reduced burst durations. In C, note delay of the time of bursting until cessation of the current, followed by immediate resumption of normal bursting form and rate. (Tazaki and Cooke, 1979a, figs. 5B, 11.) (B) Spontaneous bursting in an isolated *Homarus* ganglion segment containing a single large-cell soma. The trace is a penwriter record from Cell 3 isolated by three ligatures at distances of ca. 1.5 mm from the soma (see diagram). The activity takes the form of pacemaker potentials giving rise to driver potentials with superimposed impulses. The source of the small deflections on the pacemaker potential is undetermined. Maximum hyperpolarization, -55 mV. (Tazaki and Cooke, 1983c, fig. 9.) (C) Spontaneous activity of Cell 1 isolated from *Carcinus maenas* for 1 day in culture (resting $V_m = -34$ mV). Trace shows small deflections riding on top of the driver potential that appear to be aborted spikes. (Saver *et al.*, 1999, fig. 9A.)

responsiveness to stretch. For an isolated heart, the plots of heart rate vs. perfusion pressure and the extent of contraction vs. perfusion pressure can be superimposed, and they

increase along a hyperbolic curve (Maynard, 1960; see also Kuramoto and Ebara, 1984a, 1985). A possible role of hypoxia in governing heart rate has been proposed

(Wilkins, 1993). Although it is likely that stretch depolarizes the neuronal processes, as is well documented for crustacean stretch receptors (*e.g.*, Eyzaguirre and Kuffler, 1955), direct recordings of CG neuron responses to stretch of heart muscle appear not to have been obtained. In decapod hearts opened ventrally to expose the CG (Fig. 1A), contractions of the quiescent heart muscle can be elicited by probing sites in which CG dendrites are embedded (Alexandrowicz, 1932; Cooke, 1962; Hartline, 1967). Electrophysiological monitoring of the CG confirmed the absence of activity in the quiescent *Homarus* heart (Hartline, 1967).

The CG, after removal from the heart, exhibits the spontaneous, continuous, rhythmically recurring bursts of impulse activity described above. Intracellular recordings from the small, posterior neurons have consistently shown a slowly depolarizing pacemaker potential that develops after the post-burst hyperpolarization and leads to the initiation of the next burst (Fig. 6A) (Tameyasu, 1976; Tazaki and Cooke, 1979a, 1983c). In *Homarus*, a pacemaker potential is not usually seen in undamaged large cells, the DP and burst being initiated in them by synaptic driving mediated by impulses of the small-cell axons.

The question raised by the contrast in behavior of unstretched hearts and isolated ganglia is whether stretch-induced depolarization of dendrites and collateral processes normally induces bursting activity of the ganglion, while in isolated ganglia, the pacemaker potentials that functionally replace this stretch response in fact represent an injury current. It is possible that differences in the prominence of pacemaker potentials in recordings from large cells, even in the same species on the same equipment in the same laboratory (*e.g.*, Tazaki and Cooke, 1979a; Benson, 1980; Berlind, 1982), represent differences in techniques of ganglion isolation or of intracellular electrode penetration. During the hyperpolarization following bursts and the interburst interval, the gradual reduction in conductance to K^+ or in hyperpolarizing electrogenic active transport (Livengood and Kusano, 1972; Livengood, 1983) can lead to net depolarization as the balance of currents unmask an outward injury or "leak" current mediated by nonspecific ionic conductances. The greater prominence of pacemaker potentials in small cells, and thus their role in initiating bursting, may be related merely to their smaller size. Observations thus far available provide no evidence for the participation of a hyperpolarization-activated cationic current, such as I_h , associated with pacemaker depolarization in several vertebrate pattern-generating neurons (reviews: Kaupp and Seifert, 2001; Santoro and Tibbs, 1999). Such a conductance would be expected to cause "sag" in the voltage responses to hyperpolarizing current. The opposite—increasing hyperpolarization indicating a decrease in conductance—occurs, as seen in Figure 4A.

Experiments in which segments of the ganglion that include single large cells are isolated have shown that such

cells "spontaneously" produce rhythmical DPs (with superimposed impulse trains, if a trigger zone remains) when a pacemaker (injury?) current provides depolarizing drive (*e.g.*, Connor, 1969; Berlind, 1982; Tazaki and Cooke, 1983a, c). Cell 3 of *Homarus* has more often shown recurrent DPs after ligaturing than have Cells 1 or 2 (Fig. 6B), perhaps because it has three axons and requires three ligatures rather than one, which results in greater injury current. In cells that do not show a pacemaker (or injury) current, rhythmical activity appears if a sustained depolarizing current is passed into the neuron, or, often, if TEA is added to reduce residual K^+ current (Berlind, 1982). Recently, rhythmic "bursting" has been recorded from large cells isolated into primary culture from a crab (*Carcinus maenas*) CG (Saver *et al.*, 1999) (Fig. 6C). The observations on reduced preparations demonstrate that each of the large cells, at least, is intrinsically capable of generating rhythmic, patterned bursts given a nonspecific excitatory drive (*e.g.*, depolarization by injury current).

Synaptic Interactions

Excitatory chemically-mediated synapses

Studies such as those described earlier (*Electrophysiological anatomy*) confirmed that impulses of the posterior small-cell axons, which do not exit the ganglion, initiate activity of the large motor neurons and can thus be considered to have a pacemaker function (see also Mayeri, 1973a). Impulses of specific small-cell axons can be correlated with excitatory postsynaptic potentials (EPSPs) recorded intracellularly from large-cell somata (Fig. 6A) (Hagiwara and Bullock, 1957; Hartline and Cooke, 1969; Connor, 1969; Tazaki, 1971b; Friesen, 1975a, b, c; Tameyasu, 1987). In spiny lobsters, the most anterior of the small cells (Cell 6) provides a large initial EPSP, followed by one or more of reduced amplitude (antifacilitation); after a pause equivalent to the interburst interval, the next EPSP is augmented (Friesen, 1975c; Tameyasu, 1987). Synaptic interaction between large cells has been described in *Homarus* (Hartline, 1979). The observation that the bursts from small cells increase in rate and shorten when large-cell impulses (but not DPs) are eliminated by TTX applied anteriorly demonstrates an influence of large-cell activity on the small cells (Berlind, 1989). Synaptic potentials have not been observable in the intracellular recordings available from small cells. It may be that synapses occur at too great an electrotonic distance from the soma to be recorded. The possibility that non-impulse-mediated transmission occurs, as shown in the stomatogastric ganglion (Graubard *et al.*, 1983), has not been explored. Impulse-mediated EPSPs have been recorded in the large CG cells of several crab species (review: Hagiwara, 1961; Tazaki, 1967; Tazaki and Cooke, 1979a, 1983c; Berlind, 1982).

In the CG, as in crustacean ganglia generally, synaptic

interactions occur in complex neuropils formed by collateral processes and branches from axons (Alexandrowicz, 1932, 1934, 1952; Ohsawa, 1972; Aizu, 1975; Hawkins and Howse, 1978; Mirolli *et al.*, 1987; Morganelli and Sherman, 1987). By injecting procion rubine and horseradish peroxidase (markers that can be distinguished by electron microscopy) into two of the large cells, Mirolli *et al.* (1987) demonstrated that synapses occur in all combinations among small and large cells in neuropil of the crab (*Portunus sanguinolentus*) ganglion (Fig. 8A). Electron microscopy of neuropil in *Homarus* showed symmetrical synapses (Morganelli and Sherman, 1987). In both studies, all synapses among CG cells had clear-cored vesicles, similar to those reported by others at excitatory neuromuscular synapses (Atwood, 1976). Synaptic contacts are also contributed within the neuropils by the inhibitory and excitatory extrinsic regulator fibers. These have morphologically different vesicles and, in turn, differ in morphology from those of the intrinsic cell synapses.

The identity of the synaptic transmitter or transmitters among the CG neurons remains under discussion. The small-cell synaptic effect on large cells is rapid and therefore must be mediated by a fast, ionotropic transmitter-receptor interaction. Current evidence now strongly supports glutamate as the intraganglionic transmitter. Applied glutamate depolarizes large CG neurons of *Homarus* by increasing conductance to monovalent cations (Cooke, 1966). Under voltage clamp (but not space clamp), glutamate increased conductance and produced an inward current that had a reversal potential of ~ -15 mV and was dependent nearly equally on K^+ and Na^+ . Quisqualate was an even more potent agonist than glutamate, while L-aspartate was as potent as glutamate (Hashemzadeh-Gargari and Freschi, 1992).

Immunolabeling for glutamate-like reactivity in *Panulirus argus* (Fig. 7A, B) has shown a compound presumed to be glutamate in each of the CG cells and their axons. It has also revealed extensive terminations of the small cells in neuropil and in networks surrounding the large-cell somata and proximal neurites (Delgado *et al.*, 2001). Similar termination of labeled processes in the posterior of the ganglion near small cells is not reported. The demonstration of glutamate-like immunoreactivity in the axons and their terminations on the heart muscle supports the electrophysiological evidence for glutamate as a transmitter at the neuromuscular junctions (Benson, 1981). Additional evidence is the detection, by high-pressure liquid chromatography, of glutamate in extracted CG of the isopod *Bathynomus doederleini* and, in lower amounts, in heart muscle (Yazawa *et al.*, 1998). Modulation of CG activity by the extrinsic fibers and neurohormones is further discussed below.

Electrotonic coupling

An important element ensuring the coordinated bursting of the motor axons from the CG is the presence of electrotonic coupling among all of the neurons (Watanabe, 1958; Hagiwara *et al.*, 1959; Watanabe and Takeda, 1963; for small cells in crab CG, see Tazaki and Cooke, 1979a). The coupling passes slowly changing potentials effectively, but not rapidly changing ones such as impulses (the case of synchronization of large-cell impulse firing in the crabs *Portunus* and *Callinectes* was mentioned above). The coupling results in the effective spread of both pacemaker and driver potentials (or of imposed current) among the nine neurons, helping to ensure coordination of their bursting activity (Figs. 3C, D; 6A). Although electrophysiological recording demonstrates effective electrotonic coupling between large and small cells, dye coupling has not been demonstrated. Lucifer yellow injection into any of the large cells in *Portunus* resulted in the appearance of dye in all of the other large cells, but not in any of the small cells (Fig. 8D) (Tazaki and Cooke, 1983a). Similarly, injection of neurobiotin into any of the large cells of the *Panulirus* CG resulted in its appearance in all large cells, but not in any small cells (Delgado *et al.*, 2001). A search for images suggesting gap or tight junctions in neuropil has failed to detect them (*e.g.*, Mirolli *et al.*, 1987, in *Portunus*; Morganelli and Sherman, 1987, *Homarus*). Areas of membrane close apposition are observed among fine processes in neuropil and may serve as electrotonic junctions (Fig. 8A). The location of electrotonic connections on the small collateral processes would account for the low-pass filtering of electrotonic transmission observed electrophysiologically. These studies found that axo-axonic close appositions occur between the small-cell axons (Fig. 8B, C). Such appositions have also been described in CG of *Panulirus* (Ohsawa, 1972) and *Squilla* (Irisawa and Hama, 1965; Watanabe *et al.*, 1967a). In all of these species, the axo-axonic close appositions occur only between axons of small cells or pacemaker neurons.

Mechanisms for Modulation of Heart Function in Response to Physiological Demands

Crustacean heart muscle is similar to other crustacean muscle in being striated and innervated by multiple distributed boutons of the motor axons of most or all of the five CG motoneurons (Fig. 7B) (Anderson and Smith, 1971; Kuramoto and Kuwasawa, 1980). Heart muscle fibers are electronically coupled, end-to-end, unlike other muscle in the animal (*e.g.*, Anderson and Cooke, 1971). Neural activation of heart muscle snares most of the characteristics of neuromuscular physiology observed in other crustacean muscles: repetitive impulses produce EPSPs that facilitate extensively, and there is spatial as well as temporal summation of responses to impulses arriving from different

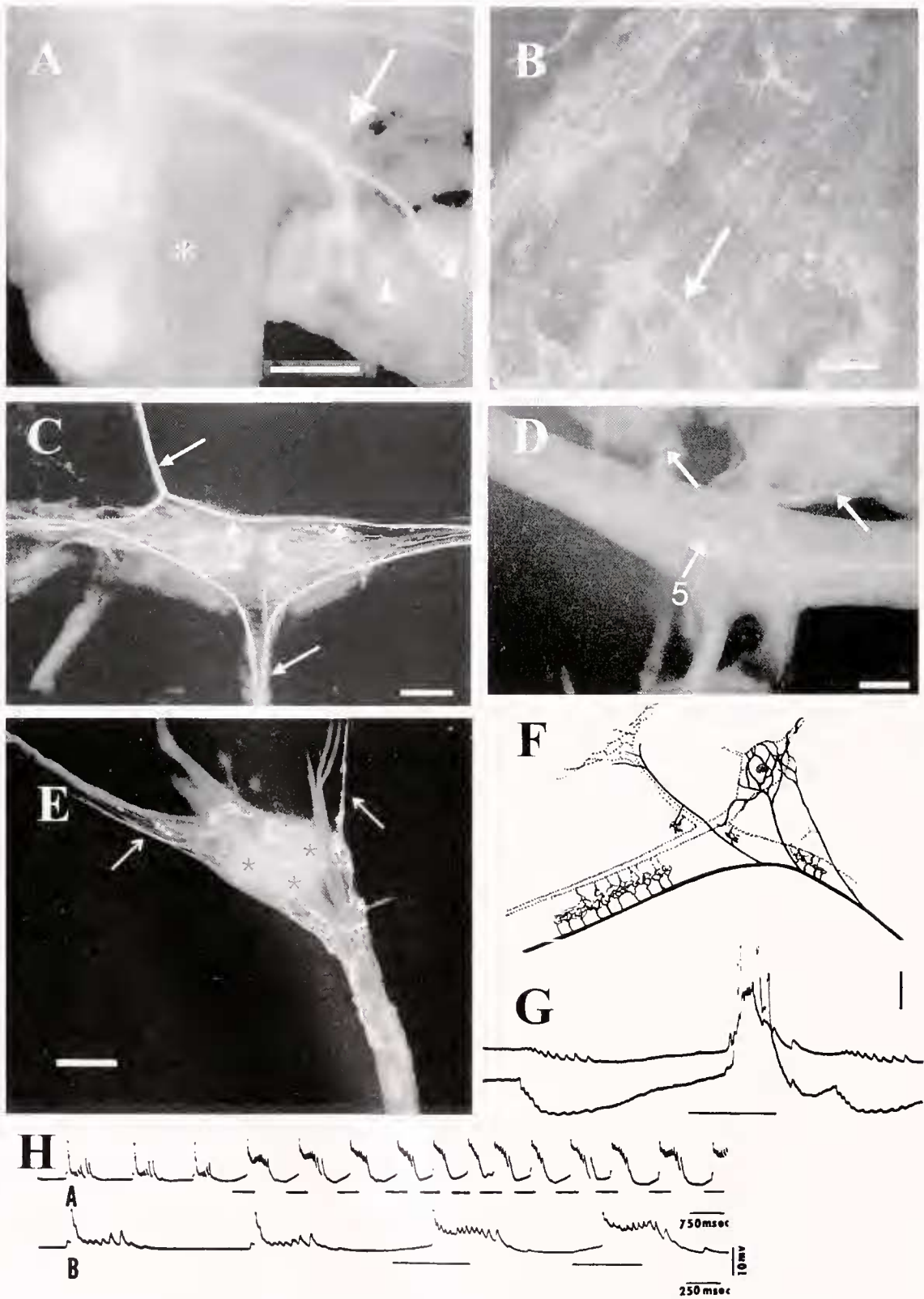


Figure 7. Chemically mediated synaptic interaction and effects of extrinsic regulator fibers. (A) Glutamate-like immunoreactivity in large cells of *Panulirus argus* cardiac ganglion. Fluorescence was present in the somata and neurites, but some major axons within the ganglion did not exhibit high levels of staining (asterisk marks site of an axon visible in neurobiotin fill, not shown). Calibration: 100 μm . (Delgado *et al.*, 2001, fig. 7B.)

motorneurons (van der Kloot, 1970; Anderson and Cooke, 1971; Kuramoto and Kuwasawa, 1980; Benson, 1981; Florey and Rathmayer, 1990; Brown, 1964a, b; for a summary and references to recording from crustacean and other arthropod hearts, see table 1 in Anderson and Cooke, 1971). The strength of contraction depends mainly on the degree to which the facilitated and summed EPSPs produce a sustained depolarization of the muscle (Orkand, 1962). Hence, the heart rate and strength of heart contractions are exquisitely sensitive to the rate, intraburst frequency, and also the patterning of impulses of each of the CG motorneurons. The effects of modulators on heart function can be largely explained by their effects on CG output.

Extrinsic fibers from the CNS

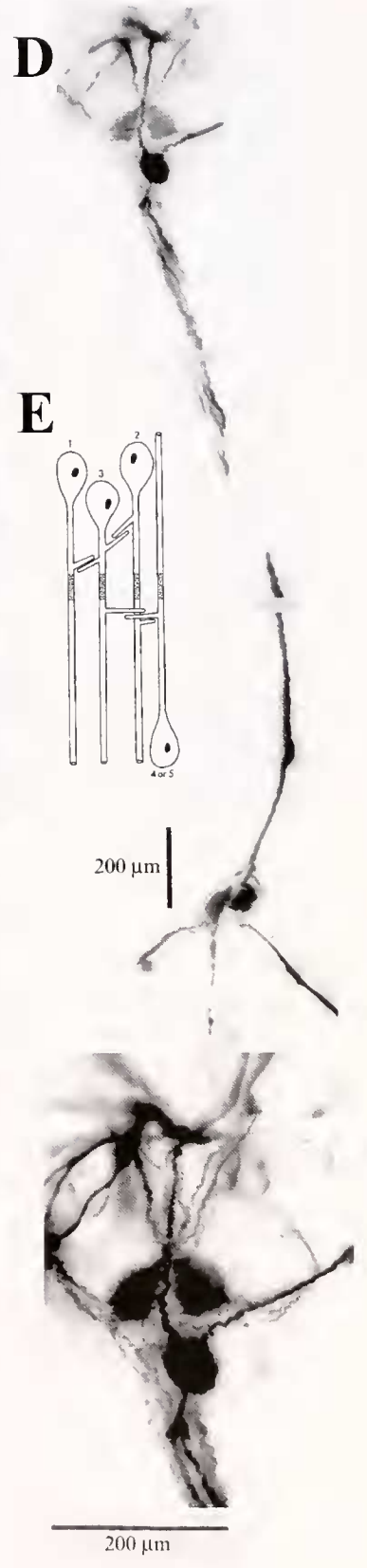
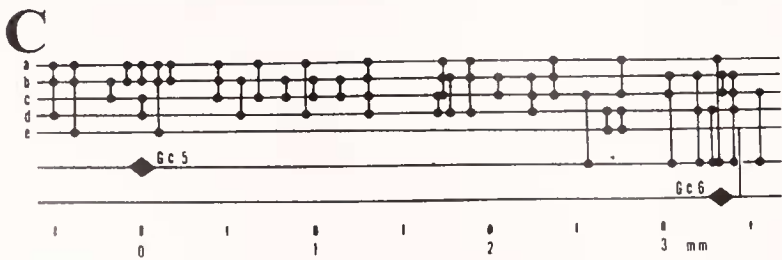
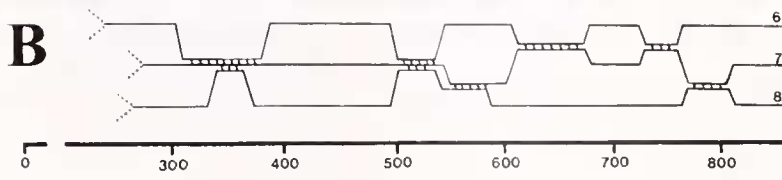
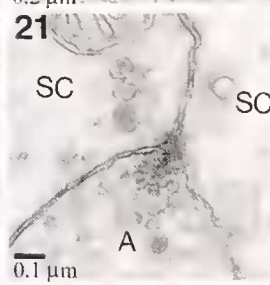
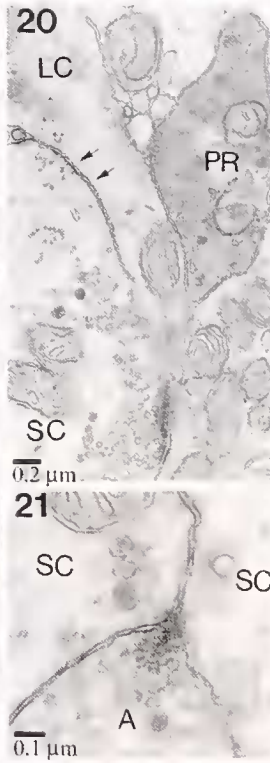
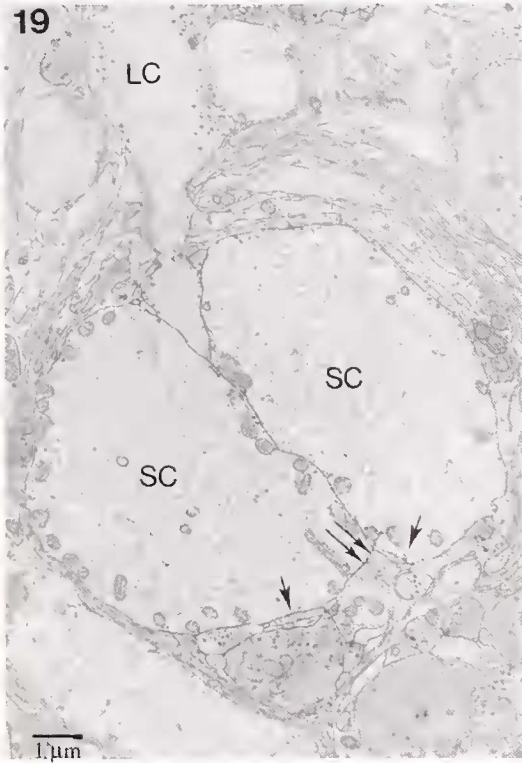
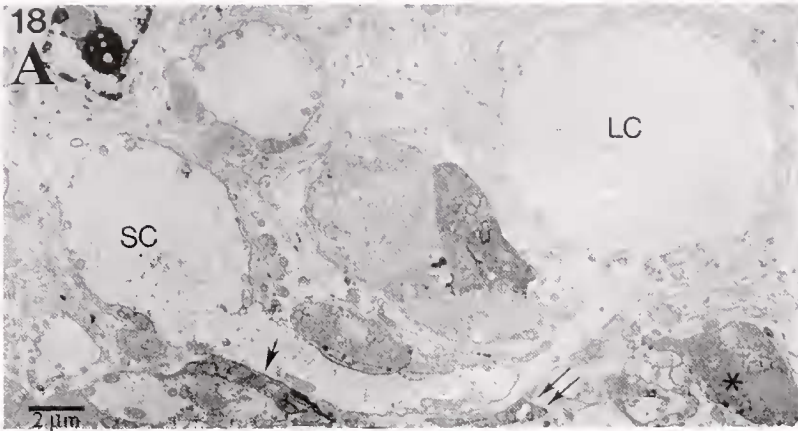
Numerous studies of semi-intact preparations have demonstrated the effects of electrically stimulating the extrinsic fibers to the heart (see Maynard, 1961, for references to early studies). Implanted electrodes and heart monitoring in lobsters (*Homarus*) have shown that the extrinsic nerves modify heart rate appropriately in relation to behavioral demands. For example, exercising on a treadmill accelerated the heart rate; bradycardia accompanied startle responses (Guirguis and Wilkens, 1995). In crayfish and lobsters the inhibitor and the two accelerator extrinsic nerves can be stimulated separately before they combine to enter the heart to reach the CG as the dorsal nerve (Fig. 1B, C). If the regulatory nerves are stimulated together, inhibition predominates.

Responses to stimulation of inhibitory fibers. The effectiveness of stimulation of the inhibitor depends on the rate of stimulation over a range of ~3–40 stimuli per second (review: Maynard, 1961; Cooke, 1962, in *Homarus*), with rapid, complete arrest for tens of seconds observed for rates of 20/s or higher. Inhibition is generally followed by a rebound acceleration of heart rate.

Stimulation of the extrinsic regulator nerve during intracellular recording from large CG neuronal somata produces postsynaptic potentials following, one for one, the stimuli to the axon, and repetitive stimulation causes the expected slowing of bursting (Fig. 7G). Inhibitory stimulation tends to move the membrane potential toward the most hyperpolarized levels observable during spontaneous bursting (Otani and Bullock, 1959; Shimahara, 1969a; Matsui *et al.*, 1973; Watanabe *et al.*, 1968, in *Squilla*). As first seen at the neuromuscular junction (Fatt and Katz, 1953), the reversal potential for the inhibitory conductance is close to the prevailing membrane potential and involves primarily an increase in conductance to Cl^- (Shimahara, 1969a). As for peripheral inhibition, γ -amino butyric acid (GABA) is a candidate for the inhibitory transmitter. In support of the earlier study (Shimahara, 1969a), voltage-clamping of large cells of *Homarus* indicated that GABA application increases conductance to Cl^- (Kerrison and Freschi, 1992).

A study of GABA-like immunoreactivity in *Panulirus* CG (Delgado *et al.*, 2001) found labeling in a single fiber entering the ganglion in the dorsal nerve from each side. These formed extensive processes in neuropil throughout

(B) Glutamate-like immunoreactive fibers could be followed over the surfaces of some muscles, where they often coursed in pairs (arrow) or triplets prior to diverging and producing a fairly uniform innervation of the muscle fibers. Calibration: 40 μm . (Courtesy J. Delgado.) (C) GABA-like immunoreactivity. A single fiber approaches the ganglion from each side *via* the dorsal nerve, bifurcates just before (lower arrow) or after (upper arrow) entering the trunk, giving rise to one large-caliber fiber directed toward the more rostral portions of the ganglion (left) and another directed caudally. Fine branches of both large GABA-immunoreactive fibers produced two spherical baskets of varicose fibers (arrowheads). Calibration: 100 μm . (Delgado *et al.*, 2001, fig. 2B.) Compare Fig. 1D. (D) Two GABA-like immunoreactive fibers that could be traced to their entry *via* their respective dorsal nerve in the anterior portion of the ganglion are seen to give rise to a spherical network of varicose fibers surrounding Cell 5. Additional branches exit the ganglion laterally (arrows) to adhering muscle bundles (compare Fig. 7F). Calibration: 100 μm . (Delgado *et al.*, 2001, fig. 3C.) (E) Tyrosine-hydroxylase-like immunoreactivity (TH-li) in crab (*Callinectes sapidus*) cardiac ganglion; the three motor neurons of the anterior end were filled with neurobiotin and labeled with rhodamine-avidin. A single TH-li fiber entered *via* each dorsal nerve (arrows) and gave rise to sparse varicose networks in the region of the motorneurons. Calibration: 100 μm . (Courtesy of T. Fort and M. W. Miller.) (F) Alexandrowicz' drawing of contacts by a dorsal nerve System 1 fiber with a large cell. A basket of processes surrounds the soma, processes accompany a dendrite, extensive contact occurs with collaterals in neuropil, and a branch extends onto heart muscle. (Alexandrowicz, 1932, Text-fig. 20; by permission of Company of Biologists, Ltd.) Compare Fig. 7D. (G) Intracellularly recorded responses to spontaneous activity of the extrinsic inhibitory fibers recorded in *Panulirus japonicus* cardiac ganglion. KCl electrodes intracellularly to Cells 5 (upper) and 2 (lower trace). Trains of inhibitory postsynaptic potentials (IPSPs) corresponding between both cells are seen before and after the intrinsic burst. Net hyperpolarizations of the soma membrane with depolarizing IPSPs in the upper cell correspond to large net hyperpolarizations with hyperpolarizing IPSPs in the lower cell. Calibration: 5 mV, 500 ms. (Matsui *et al.*, 1973, fig. 2; reprinted with permission from Elsevier Science.) (H) Effects of repetitive stimulation of an acceleratory extrinsic regulator fiber on spontaneous burst activity, recorded intracellularly from a large cell of *Panulirus japonicus* cardiac ganglion. A, 30 Hz; B, 50 Hz during periods marked by the bars. (Shimahara, 1969b, fig. 2.)



the ganglion, including a pericellular network around large-cell somata (Fig. 7C, D). Processes also were seen to ramify into the heart together with the large-cell dendrites presumed to be mediating stretch sensitivity. This morphology strikingly matches the description by Alexandrowicz (1932) of the System I fibers that he proposed were inhibitory (Figs. 1D, 7F).

In electron microscopy studies (Morganelli and Sherman, 1987, *Homarus*; Mirolli *et al.*, 1987, *Portunus*), synapses that were found extensively in neuropils throughout the CG had terminals with vesicles similar to those associated with inhibitory synapses on crustacean muscle (Atwood, 1976). These anatomical observations provide a structural basis for the observation that inhibitory regulatory fibers are more effective than excitatory ones (Maynard, 1961), as well as for the greater prominence of postsynaptic effects of inhibition as observed from CG somata recording.

Responses to stimulation of accelerator fibers. In his 1932 study, Alexandrowicz described a pair of smaller fibers ("System II") accompanying the larger fiber ("System I," now confirmed as inhibitory) in the dorsal nerve (Fig. 1B) bringing extrinsic innervation to the CG. He suggested that these represented the acceleratory inputs. They show sparse terminations in the ganglion and send processes into the cardiac muscle.

Effects of stimulation of the accelerator fibers have been difficult to characterize because the effectiveness of stimulation appears to fatigue rapidly, especially for stimulation of the more posterior of the two fibers. Bouts of stimulation resulted in slowly developing depolarization recorded in

large CG neurons of *Panulirus* (Fig. 7H) (Shimahara, 1969b; see also Terzuolo and Bullock, 1958). Similar responses were seen in *Squilla* (Watanabe *et al.*, 1969) and in the isopod *Ligia exotica* (Sakurai and Yamagishi, 1998).

A recent advance toward resolving the nature of the transmitters for the accelerator fibers is the observation of immunoreactivity to an antibody raised against tyrosine hydroxylase in one of the two fibers reaching the CG from the CNS on each side in *Callinectes* (Fig. 7E) (Fort and Miller, 2001). Tyrosine hydroxylase catalyzes the addition of a hydroxyl to tyrosine to form dopamine. Together with earlier observations of the presence of a catecholamine in CG tissue (Ocorr and Berlind, 1983) and the excitatory effects of dopamine on the CG (see further below), the observations implicate dopamine as the transmitter for one of the accelerator fibers. Which of the two fibers shows the immunoreactivity is still unresolved.

Among many agents that have acceleratory effects on the CG, possibly the strongest candidate transmitter for the second fiber is acetylcholine (Freschi and Livengood, 1989; Sullivan and Miller, 1990; see further below). 5-Hydroxytryptamine (5HT) has been eliminated as a transmitter, at least for the more anterior of the accelerator fibers in *Homarus*. Response to 5HT could be blocked by previous application of D-lysergic acid diethylamide (LSD) without blocking the effects of nerve stimulation (Cooke, 1966). Responses to stimulation of the more posterior accelerator nerve were weak or absent, and hence no conclusion about inhibition by LSD was possible. Glutamate is unlikely, as

Figure 8. Morphology of synaptic and electrotonic contacts. (A) Contacts among neurons of *Portunus sanguinolentus* cardiac ganglion: Electron micrographs from Mirolli *et al.*, 1987. (18) Contact between the collateral process of a small-cell axon (SC) and that of an anterior large cell (LC). SC and LC processes are distinguishable by the density and distribution of their cytoskeletal elements. A process from a different anterior LC injected with procion rubine (PR) contacts the SC collateral process (arrow), and another (double arrows) contacts the collateral process of the LC. Asterisk marks another part of the PR-labeled process. (19) Contacts between two SC axons and between them and the collateral process of a LC. Other processes (arrows) are also in contact with the two SC axons. (20) Higher magnification of the contact between the LC and SC collateral processes shown in 18. Note the absence of glia at the region of contact. Arrows point to possible membrane close appositions between the two collaterals and that of the LC and a PR-stained process. (21) Synapse between a process with vesicles typical of intrinsic cell processes and two small axons (SC). (B) Schematic drawing of the chain of contacts made by three small-cell axons of the *P. sanguinolentus* ganglion as reconstructed from a complete series cut for light microscopy. The horizontal scale shows the distance (μm) of the contacts found in this series from a reference section in the anterior end of the trunk. (Mirolli *et al.*, 1987, fig. 17.) (C) Schematic representation of the side-junctions [close contacts] (vertical lines) between axons in the stomatopod (*Squilla oratoria*) cardiac ganglion in a section of the ganglion with ganglion cell (Gc) 5 and Gc 6. Lines a-e represent axons passing in the main trunk. (Watanabe *et al.*, 1967a, fig. 1; reproduced by copyright permission of The Rockefeller University Press.) (D) Reversed photomicrographs of a *P. sanguinolentus* cardiac ganglion injected with Lucifer yellow. The trunk was ligatured (central undyed portion), then Cell 3 in the anterior and Cell 4 in the posterior portion were intracellularly injected. In the photomontage of the entire ganglion, Cells 1 and 2 in the anterior cell group (top) and 5 in the posterior group are dimly visible as a result of dye transfer. Dye fails to diffuse within 100 μm of the ligature. Below: Detail of anterior cell group, overexposed to show web of fine processes near somata and parallel-running anteriorly directed processes. The four posterior small neurons are never visualized in such preparations. (Tazaki and Cooke, 1983a, fig. 1a, b; by permission of Springer-Verlag CmbH & Co. KG.) (E) Schematic showing sites of initiation of the synchronous motorneuron impulses (stippling) in the *P. sanguinolentus* ganglion (I. M. Cooke, unpublished.)

no glutamate-like immunoreactivity was detected in the dorsal nerves of *Panulirus* (Delgado *et al.*, 2001).

Neurohormonal modulation

Neurohormones of the pericardial organs. Alexandrowicz (1953) recognized that the webbings of neural tissue spanning the openings of the branchial sinuses in crabs were neurohemal structures and named them pericardial organs (POs). Given their location in the path of hemolymph returning from the gills and about to enter the heart, functions in modulating heart and circulatory performance were anticipated and soon established (Alexandrowicz and Carlisle, 1953). Homologous structures of lobsters are the ligamental nerve plexuses, now referred to as POs (Alexandrowicz, 1932, 1953; reviews: Cooke and Sullivan, 1982; Chaigneau, 1983). Neurohormones found in POs include the amines 5HT, dopamine (predominant in crabs), and octopamine (predominant in lobsters, Sullivan *et al.*, 1977). Peptides that have been identified include proctolin (Sullivan, 1979), crustacean cardioactive peptide (Stangier *et al.*, 1987; review, Dirksen, 1994) in crabs, and one or more FMRF-amide-related peptides (Trimmer *et al.*, 1987; Mercier *et al.*, 1993). Most of these are also found elsewhere in the nervous system but have effects on central pattern generators and at neuromuscular junctions at concentrations consistent with their role as circulating neurohormones.

Neurohormonal effects on the heart. It would not be possible here to review the extensive literature from at least 80 years of experiments on the effects of tissue extracts, putative neurotransmitters, hormones, and pharmacological agents on crustacean hearts. More recent studies examining effects of PO extracts and hormones on nearly intact and *in situ* hearts suggest that each has subtly differing sites and modes of action in altering heart performance (Florey and Rathmeyer, 1978; Kuramoto and Ebara, 1984a, b, 1988, 1991; Krajniak, 1991; Yazawa and Kuwasawa, 1992; Mercier and Russenes, 1992; Wilkens and Mercier, 1993; Wilkens *et al.*, 1996; Saver and Wilkens, 1998; Saver *et al.*, 1998). Hormones may have differential actions on the valves governing hemolymph distribution to different parts of the animal *via* the several arteries (Kuramoto *et al.*, 1992), on alary muscles contributing to distension and refilling of the heart, on tension development by heart muscle, and on the neuromuscular junctions as well as on the CG. A broad generalization that is increasingly supported as further experiments are done is that each of the neurohormones orchestrates a subtly different coordinated response of the circulatory, respiratory, and probably other systems to homeostatic demands—as, for example, to anoxia, osmotic stress, trauma, and the like. In general, the PO neurohormones produce slowly developing and long-lasting increases in heart rate and strength of contraction.

Neurohormonal effects on the cardiac ganglion. Many, but clearly not all, of the effects of PO neurohormones on intact heart preparations can be accounted for by the effects observable on the efferent output from isolated CG. These hormones act on isolated CG preparations, generally increasing burst rate and usually increasing the frequency and number of impulses as well as the duration of the bursts. In an effort to distinguish sites and mechanisms of action within the CG, a localized droplet of hormone-containing saline was applied to a lobster CG held on an array of electrodes in oil (Fig. 2B) (Cooke and Hartline, 1975). Defining excitation of a neuron as an increase in the *average* firing frequency of one of its axons, the study found that sensitivity to neurohormones was greatest at the impulse-initiating zone and confined to the nonspiking, integrative, DP-generating, initial axon segment of each of the neurons; the somata themselves were not sensitive.

The droplet technique and the limited region of cell sensitivity made it possible to observe how neurohormone stimulation of a single cell altered the performance of the otherwise undisturbed cardiac ganglion (see Fig. 2 and legend). Stimulation of individual axons that more than doubled their average firing rate was accommodated into the bursting pattern by correspondingly much greater increases in the rate at which those axons fired within the burst (as in Fig. 2C). Analysis of the impulses of individual axons revealed that those not exposed to the hormone in their sensitive region retained a relatively unchanged *average* firing frequency. The overall burst rate was altered only if an application changed the average firing frequency of the acting pacemaker (small-cell) axon. The robustness of the integrating ganglion as a rhythmic heartbeat activator was emphasized by these observations: despite major perturbation of individual axon frequency, the rhythmical output of tightly grouped motor impulses was never disrupted.

The difference in effects of the neurohormones when the substances are applied regionally to primarily act on sensitive regions of small cells or large cells adds additional evidence for differences in the intrinsic properties of these cells and, particularly, in the nature of their DPs (proctolin: Miller and Sullivan, 1981; Sullivan and Miller, 1984; octopamine: Benson, 1984; dopamine: Miller *et al.*, 1984; Berlind, 1998 [see Fig. 9E], 2001a, b; 5HT: Kuramoto and Ebara, 1988; Kuramoto and Yamagishi, 1990; Berlind, 1998). An example is provided by observations on the effects of proctolin applied to the isolated *Homarus* CG (Miller and Sullivan, 1981; Sullivan and Miller, 1984). Application to small cells increased the duration and firing frequency of small-cell axons, implying an underlying increase in the duration and amplitude of the small-cell DPs. This effect was rapid (5–10 s), and it could be rapidly reversed. Application of proctolin limited to large-cell sensitive regions (Fig. 9A) caused a slowly developing increase in burst frequency accompanying a depolarization of up to

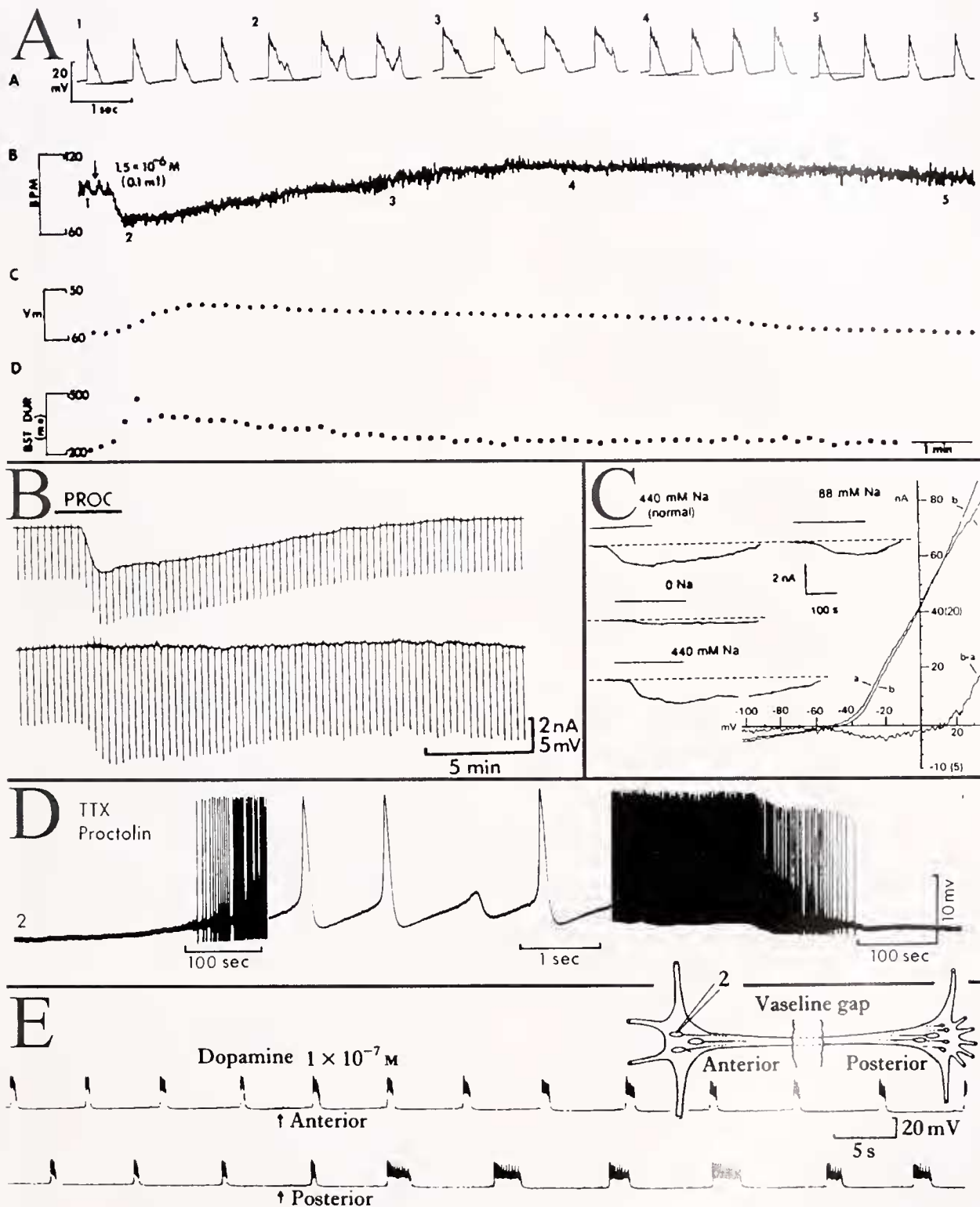


Figure 9. Neurohormonal effects on the isolated cardiac ganglion. (A–D) Responses to proctolin. (A) Intracellular recording from motoneuron 1 ($V_m = -59$ mV) demonstrates the time-dependent proctolin effects. Samples of burst activity (1–5) were taken at the times indicated on the continuous plot of instantaneous burst frequency (B). A total of 14 min is shown. Time 1 is control. Within 10 s of application of a pulse of proctolin (1.5×10^{-6} M, 100 μ l), burst frequency began to decline, (B) reaching a minimum of 65 per min after about 25 s. Concurrently, the ganglion exhibited a period of double bursting (Time 2). Considering each doublet as a

8 mV. By contrast with responses of the small cells, the response to a brief pulse of proctolin developed over minutes and lasted for tens of minutes.

For proctolin, there are observations suggesting its membrane-level mode of action. When proctolin was applied to *Homarus* large cells isolated by ligaturing and TTX treatment (Fig. 9C), the resulting depolarization could induce rhythmic driver potentials in previously quiescent cells (Sullivan and Miller, 1984). Tests of the cell input resistance (Fig. 9B) showed that proctolin increased resistance, and ionic substitution experiments indicated that this represented primarily a decrease in residual K^+ conductance. Removal of Na^+ decreased the magnitude of proctolin depolarization, which these authors interpreted as confirming that depolarization normally resulted from increased effectiveness of a "leak" conductance, for which Na^+ was the dominant ion in depolarizing the membrane. Similar observations (Fig. 9C) were made by Freschi (1989), but interpreted as representing activation of a Na^+ current.

The actions of crustacean cardioactive peptide and the FMRamide-related peptides on isolated cardiac ganglia seem not to have been reported.

Are there intrinsic neuromodulators in the cardiac ganglion? There appear to be neurohumoral modulatory effects that arise within the CG and that supplement the actions of the fast-acting synapses. This possibility is raised by obser-

vations of the effects of acetylcholine, a catecholamine, and nitric oxide (NO) and by indications that these agents are present in the ganglia. Involvement of ACh is implied by the staining of neuropil showing the presence of acetylcholinesterase (E. Maynard, 1971); the ability of CG tissue to synthesize ACh (Sullivan and Miller, 1990); and sensitivity of the ganglia to ACh, with evidence for both nicotinic and muscarinic receptors (Freschi and Livengood, 1989). Responses of the CG to perfused ACh closely resemble those described above for proctolin (Freschi and Livengood, 1989; Sullivan and Miller, 1990). Synthesis of catecholamines by the CG and catecholamine fluorescence in large cells of *Homarus* have been reported (Ocorr and Berlind, 1983). However, tyrosine-hydroxylase-like immunoreactivity was not observed in cells of the *Callinectes* CG (Fig. 7E) (Fort and Miller, 2001). Large, dense-cored granules are present together with the predominant clear-cored vesicles in some neuropil synaptic profiles, suggesting that a catecholamine could be co-localized with another transmitter (Morganelli and Sherman, 1987). The CG is responsive to low concentrations of dopamine and octopamine, as mentioned above. The complexities of the responses to pharmacological agonists and antagonists of monoamine receptors of vertebrates when tested on the *Homarus* CG leave open the possibility of intrinsic neuromodulators (Berlind, 2001a, b). Recently, nitric oxide synthase has been

single burst, there was a coincident peak in burst duration (D). (C) Maximum V_m of Cell 1: the membrane potential underwent a slow depolarization that began about 15 s after application and became maximal after 1.5 min. Maximum frequency was obtained ~ 7 min after application of proctolin (Time 4). Burst frequency exceeded the control rate for the ensuing 7 min, returning to the control frequency concurrently with motor-neuron repolarization (Time 5). (D) Burst duration: a peak during the period of double bursting corresponded with the time of minimal burst rate. (Sullivan and Miller, 1984, fig. 2; reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.) (B) Manual voltage-clamp records demonstrate that proctolin effects a net inward current and an increase in the membrane input resistance. Transected Cell 2 ($V_m = -40$ mV) in saline containing TTX (3×10^{-7} M) and cadmium (4×10^{-4} M). The slow depolarizing response was continuously canceled by passage of inward current through a second electrode (top record). Additional constant-current hyperpolarizing test pulses applied throughout the response produced voltage deflections of increased magnitude (lower record), indicating an increase of the membrane input resistance [in this example by 50%]. (Sullivan and Miller, 1984, fig. 9B; reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.) (C) Current-voltage plot of responses to slow (ramp, -140 to $+40$ mV in 44 s) changes of voltage under two-electrode voltage clamp of a ligated anterior large cell of the *Homarus* cardiac ganglion before (curve a) and in the presence of proctolin ($1 \mu M$, curve b). Curve b-a is the subtracted or proctolin-induced current. Records show reversible reduction in inward current observed (holding voltage at resting potential) during $1\text{-}\mu M$ proctolin perfusion (bars) in saline having reduced Na^+ . (Freschi, 1989, fig. 4A, B; reprinted with permission from Elsevier Science.) (D) Intracellularly recorded responses of an anterior *Homarus* cardiac ganglion cell to proctolin. Spontaneity of the ganglion has ceased as a result of inclusion of 3×10^{-7} M TTX in the perfusate. At the start of the record, a 0.1-ml pulse of 10^{-7} M proctolin was introduced to the continuing perfusion stream (1 ml/min). Gradual depolarization (3 mV from -50 mV) leads to a sustained series of driver potentials (seen at increased chart speed in the center part of the record) and eventual return to quiescence. During the response, input resistance increased 30%. The duration of the record shown is 10.5 min. (K. Tazaki in Cooke, 1988, fig. 5; reprinted with permission from Elsevier Science.) (E) Dopamine effects on spontaneous bursting of the isolated crab (*Portunus sanguinolentus*) ganglion. All traces were recorded from a cell body at the anterior end of the ganglion, but any of the five large neurons would have shown a similar pattern of activity and an identical record of action potentials. The pair of traces shows the response to application of a $50\text{-}\mu l$ pulse of 10^{-7} M dopamine (arrows) to the anterior end of the ganglion (top trace of each pair) only or to the posterior end (lower trace) only. For all applications, burst duration is increased, but responses are greater for applications to the posterior region including the small cells. (Miller *et al.*, 1984, fig. 5; by permission of Company of Biologists, Ltd.)

localized in all nine neurons of a crab (*Cancer productus*) CG (Labenia *et al.*, 1998). This finding may suggest a role for amines and NO in modulating CG bursting activity via metabotropic receptors rather than via ionotropic receptors such as those of the fast excitatory synapses. A further question is whether the extrinsic regulatory fibers play a role in governing such neuromodulation.

Cellular mechanisms of neuronal modulation. Effects of the neurohormonal modulators have in common that they develop over tens of seconds and may last for tens of minutes in response to brief exposure. This time-course indicates a mode of action involving second-messenger-mediated modulation of ionic conductances. In support of this type of action, pericardial organ extracts were found to increase cAMP levels in *Homarus* cardiac ganglia, and pharmacological manipulations that increased cAMP mimicked the effect of PO extracts (Lemos and Berlind, 1981). Manipulations expected to increase NO levels increased cGMP levels and inhibited activity of the CG, while decreasing NO synthase activity decreased cGMP levels and increased burst frequency (Labenia *et al.*, 1998). The localization of neuronal regions sensitive to the neurohormonal modulators on the proximal axon segments (Cooke and Hartline, 1975) is consistent with the suggestion that these modulate conductances controlling the pacemaker (or leak) potentials, DPs, or both.

The Relation of Driver Potentials to Other Intrinsic Potentials

The recognition from study of the lobster CG (Watanabe, 1958) that the capability for patterned or bursting impulse activity is an intrinsic property of an individual neuron has now been extended to include a number of types of vertebrate neurons as well as neurons from nearly all major invertebrate groups. A sampling of the studies describing intrinsic DP-like properties underlying patterning include—besides other crustacean systems (see below)—insects (Hancox and Pitman, 1991); molluscs (Kramer and Zucker, 1985; Hurwitz and Susswein, 1996; Perrins and Weiss, 1998); annelids (Arbas and Calabrese, 1987); and vertebrates (reviews, Cooke and Stuenkel, 1985; Llinás, 1988; Kiehn and Eken, 1998). The list of vertebrate neurons includes Purkinje cells (Llinás and Sugimori, 1980); thalamic neurons (Déschenes *et al.*, 1982; Llinás and Jahnsen, 1982); hypothalamic neurons (Legendre *et al.*, 1982); neurons responsible for lamprey swimming (Grillner *et al.*, 1991); neurons involved in respiratory rhythm generation (Rekling and Feldman, 1998); motoneurons (Hounsgaard and Kiehn, 1989, review; Hultborn, 1999); and subthalamic nucleus neurons (Beurrier *et al.*, 1999). The sustaining relevance of the studies on the CG lies in the remarkable similarity of the underlying ionic mechanisms observed in a major proportion of the pattern-forming neurons that have been analyzed.

Plateau potentials

Neurons of the decapod stomatogastric ganglion display an important variant of the intrinsic properties seen in the DPs of the CG neurons. Certain of the stomatogastric neurons exhibit plateau potentials (*e.g.*, Russell and Hartline, 1978, 1982; Dickinson and Nagy, 1983; Harris-Warrick *et al.*, 1992a; review, Hartline and Graubard, 1992; for voltage-clamp data see Golowasch and Marder, 1992; Zhang and Harris-Warrick, 1995). The term reflects their well-sustained, stable depolarized level (see Hartline, 1997, for diagnostic characteristics). A major embellishment is the control of the neuron's capability to produce plateau potentials by neurotransmitters, neuromodulators, or neurohormones (Russell and Hartline, 1984; Nusbaum *et al.*, 2001). Such modification provides different output patterning (*i.e.*, command of different behaviors) by the ganglion (reviews in Harris-Warrick *et al.*, 1992b). Modulation is also found in other crustacean pattern generators (*e.g.*, the crab ventilatory system, DiCaprio, 1997) and in vertebrate motoneurons (Hounsgaard and Kiehn, 1989; review, Hultborn, 1999) and subthalamic nucleus neurons (Beurrier *et al.*, 1999). A further embellishment on the stereotyped DP is the possibility of controlling plateau duration. Plateau potentials, like DPs (Tazaki and Cooke, 1979b), can be, and often are, terminated by hyperpolarizing current such as produced by an inhibitory synaptic potential. As with DPs, plateau potentials initiate and pattern the action potentials produced by the neuron.

Reviewing common characteristics detailed for the DPs of the CG above that have been associated with the presence of intrinsic pattern-forming capability as found in neurons from a diversity of animals and neurons, they include the following:

- Appropriate depolarization (*e.g.*, a pacemaker, synaptic potential, or imposed depolarizing current) initiates a regenerative, slowly rising, sustained (tenths of seconds to tens of seconds), depolarizing (to above impulse threshold) potential, in a majority, resulting from activation of a slowly inactivating or non-inactivating I_{Ca} . Application of TTX to silence impulse initiation as well as impulse-mediated synaptic input reveals the form of these potentials. However, DP- or plateau-like potentials involving Na^+ -inward current rather than, or as well as, I_{Ca} have been described ([for CG, see above], *e.g.*, Angstadt and Choo, 1996, leech Retzius neurons; Kim and McCormick, 1998, ferret perigeniculate neurons; Su *et al.*, 2001, rat hippocampal CA1 pyramidal cells).
- The amplitude and duration of the depolarization is shaped by the suite of K^+ conductances present and by the inactivation mechanisms of the inward currents. These include both voltage- and Ca^{2+} -dependent components. The depolarization can be terminated by

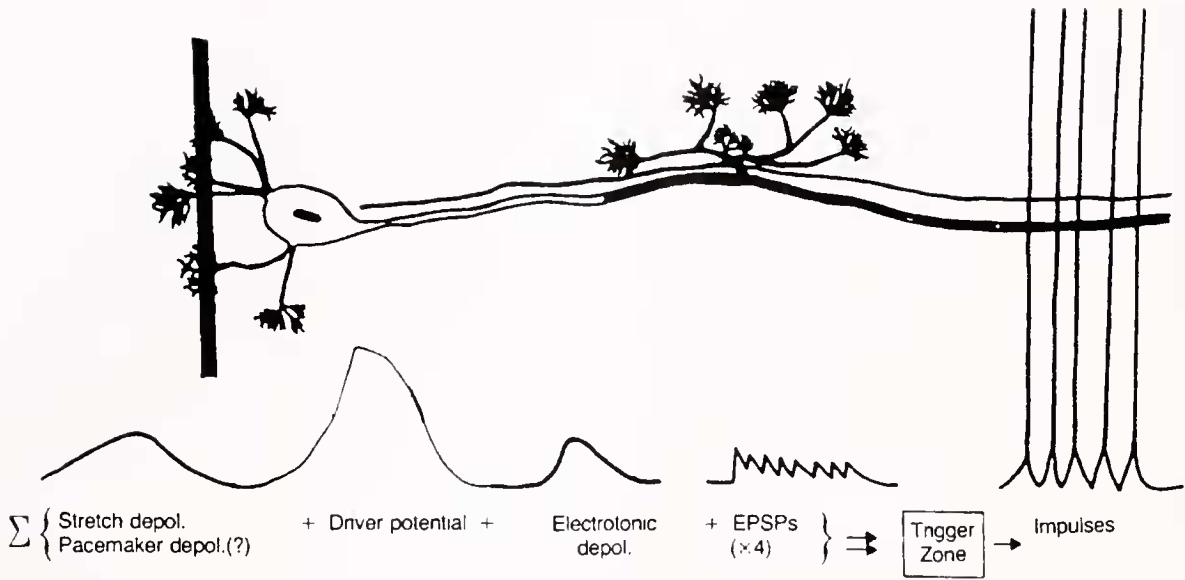


Figure 10. Anatomical segregation of function in cardiac ganglion neurons. Diagram depicting the supposed integrative region of the soma and proximal axon, which is not capable of impulse initiation. In that region, pacemaker depolarizations (if present), inputs of stretch-sensitive dendrites, and electrotonic and chemical synaptic inputs (on axon collateral processes) all sum with the regenerative, non-propagating driver potential to produce a flow of current through the membrane of the more distal axonal trigger zone. The current initiates and influences the temporal patterning of the action potentials that propagate distally. (Benson and Cooke, 1984, fig. 6; reprinted with permission from Elsevier Science.)

hyperpolarizing current (from inhibitory synaptic input or imposed current).

- The pattern-forming potentials often exhibit a relatively refractory period that may include an afterhyperpolarization. The afterpolarization may activate currents (see below) contributing toward a slow pacemaker depolarization that in turn initiates the intrinsic potential, thus contributing to rhythmic pattern generation.

Rhythmicity

Intrinsic pattern-forming characteristics combined with a pacemaking mechanism or source of sustained depolarizing drive result in rhythmic bursting or pattern formation, as exhibited in the isolated CG. One of the ionic mechanisms giving rise to pacemaker potentials is hyperpolarization-activated cationic current, now generally referred to as I_h , which has been implicated in many of the vertebrate neurons studied (reviews: Santoro and Tibbs, 1999; Lüthi and McCormick, 1998; Kaupp and Seifert, 2001). As mentioned above, I_h has not been observed in the CG motoneurons. It has been reported in a crab stomatogastric motoneuron (Kiehn and Harris-Warwick, 1992). I_h is modulated by agents that alter cyclic nucleotides such as 5HT and other amines and peptides, suggesting a mechanism by which neuromodulators influence rhythmic pattern generators.

Another current that may contribute to pacemaking is

slowly inactivating or persistent Na^+ current (I_{NaP}) (e.g., Hsiao *et al.*, 1998; Brumberg *et al.*, 2000). By contrast, in subthalamic neurons, activation of I_{NaP} changes burst firing to single-spike activity (Beurrier *et al.*, 2000). Activation or enhancement of an I_{NaP} -like current has been proposed as a mechanism of action of neuromodulators of the crustacean CG, as discussed above (Freschi, 1989; Freschi and Liven-good, 1989).

Recapitulation and Conclusions

Economy in neuronal cell numbers is achieved in arthropods by a cellular architecture that permits each cell to serve several functions. Thus, CG cells are stretch receptors, integrative interneurons, and, in the case of the large cells, motoneurons as well; they may also be endowed with the capability for spontaneity and pacemaking. The sensory and integrative functions occur in a region—the soma and initial axon segment and its collateral processes—segregated from the impulse-propagating distal axon (Fig. 10). A trigger zone converts the integrated signal to conducted, all-or-none, Na^+ -mediated impulses whose frequency and pattern reflect the form of the integrated depolarizing potential generated in the initial axon segment. The presence of a combination of ionic conductances in the integrating zone that give rise to the regenerative but graded driver potential (or in other ganglia, plateau potential) enables the neuron to produce a specific form of patterned impulses that can be

largely independent of the excitatory input. A combination of three factors—a voltage-gated Ca^{2+} conductance that is inactivated by the resulting entry of Ca^{2+} , fast and slowly inactivating voltage-dependent K^+ conductances, and finally a K^+ conductance initiated by the rise of internal Ca^{2+} —imposes a refractory period. The depth and duration of this refractory period reflects the level of previous activity. The removal or sequestering of Ca^{2+} reduces K^+ conductance, allowing increased influence of depolarizing currents (stretch, pacemaker, leak, or synaptic), and removes I_{Ca} inactivation, thus reducing threshold for the DP. Together the behaviors of these ionic conductances account for rhythmic recurrence of DPs and bursting, given general excitatory drive. The conductances also shape the amplitude and duration of DPs as a function of the rate of recurrence; they account for the reciprocity between burst rate and duration, and for the advanced or delayed phasing of the burst cycle when perturbed.

The CG shows remarkable reliability in providing rhythmic bursts of motor impulses in the face of a variety of imposed experimental perturbations and insults, and theoreticians have used it to model fault-tolerant networking (e.g., Sivan *et al.*, 1999). In the CG, reliability surely arises from the intrinsic ability for rhythmic burst formation of each neuron combined with the extensive interconnection among all the neurons that ensures coordination of their activity. Electrotonic coupling links all cells of the ganglion and passes slowly changing potentials such as stretch-induced or pacemaker potentials and DPs, and these can continue to recur synchronously and rhythmically when impulse propagation has been eliminated with TTX. Each of the cells provides fast, chemically mediated excitatory synaptic input to, probably, all other cells. While these synapses are known to be impulse-mediated, the possibility that some are also active in the absence of impulses has not been examined.

Although the similarity among the nine neurons (in the case of most decapods) perhaps provides reliability by means of redundancy, each exhibits consistent differences in the detailed pattern of its impulse bursts. In the isolated CG, small cells have prominent pacemaker depolarizations and a DP of low amplitude and long duration. This form of DP thus generates a long burst of impulses at relatively steady frequency which mediates a sustained barrage of excitatory input to the motorneurons. Large cells exhibit a larger amplitude and a shorter DP, and they produce a burst having an initial high frequency of impulse firing, efficacious in rapidly depolarizing and initiating contraction in the heart muscle fibers. Other more subtle differences among the CG neurons are also documented. Additional differences become evident in considering the modulation of ganglion output by the several neurohormones secreted from the pericardial organs. Each neurohormone produces a different change of CG output and, further, has differing

effects on small and large cells. The anatomical segregation of function within neurons is again observed with the restriction of responsiveness to neurohormones to the non-spiking initial axon segment.

The apparent simplicity of the CG has proven somewhat illusory. The above review will make obvious that there are many unresolved questions. To list a few:

- How is stretch (heart filling) transduced and integrated to influence the activity of the CG neurons? The existence and importance of stretch receptiveness is well documented and presumably provides the ability of the CG to monitor and adjust its activity to the results of its actions as well as to other external influences and thus function as an autonomous control system.
- Are there specific currents providing depolarizing pacemaker drive?
- Does non-impulse-mediated synaptic chemical transmission occur among CG neurons as, for example, in the stomatogastric ganglion (Graubard *et al.*, 1983)?
- Where and how do putative neuromodulators intrinsic to the CG such as ACh and NO act?
- What is the identity of the transmitter or transmitters released from the other extrinsic regulator fiber?
- Which conductances are involved in CG modulation by the various known and proposed neurohormones or neuromodulators?
- Which possible second messenger systems participate in modulation of CG activity, how extensive are they, and what roles do they play?

New immunoreagents invite further studies exploiting the resolving capabilities of confocal microscopy for localization of receptors and enzyme systems in the CG. The availability of voltage-clamping analyses of the ionic currents, analyses of impulse-mediated synaptic interactions, and dye-fills that provide anatomical detail invite application of modeling, as in the case of the stomatogastric ganglion, to discover missing components in the available information. Demonstration that it is feasible to isolate CG neurons in primary culture and that they retain their ability to produce rhythmical bursts and respond to neurohormones (Saver *et al.*, 1999) opens the possibility for examining some of these questions free of the complexities presented by the interactions among the neurons in even this small, "simple" ganglion.

A candidate for the most significant contribution to neurobiology of studies on crustacean cardiac ganglia would be the unambiguous demonstration that a single neuron can be endowed with the intrinsic capability of providing an output of distinctively patterned impulses in response to nonpatterned, general excitation or single stimuli. Detailed analyses of the electrophysiological underpinning of this capability are important components of the demonstration. These observations have suggested new, much simpler ex-

planations for the generation of complex neuronal patterns, including mechanisms by which neurohormonal and other modulators can alter these patterns and the behaviors they direct.

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